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TITOLO TESI:

# DETECTION AND MOLECULAR CHARECTERIZATION OF VIRUSES INFECTING *ACTINIDIA* SPP.

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# General Introduction

## Kiwifruit origin

The kiwifruit plant is native to eastern Asia and in 1900 it was just a plant growing in the hills and mountains of south-central China, between the Yangzi (Chang Jiang) and Pearl (Zhu Jiang) rivers (Datson & Ferguson, 2011) but palaeobiological studies estimate kiwifruit to be at least 20-26 million years old (Qian & Yu, 1991). One of the earliest descriptions of the plant and fruit (known then in China as mihoutao, monkey peach) was assigned to an author in the twelfth century Song Dynasty, who described kiwifruit as " found in the valleys of the mountains; it is a vine with round, pubescent leaves, which grows by climbing over trees; in shape and size the fruit resembles an egg; its skin is brown; after the first frosts, it becomes sweet and edible," as referenced by Ferguson (Ferguson, 1990b). Kiwifruit comprise more than 55 species and about 76 taxa belonging to the genus *Actinidia,* with a wide variability in fruit shape, size, colour and composition (Figure 1.1) (Ferguson, 1990a).

**Fig 1.1:** Fruit of the *Actinidia* genus showing variation in flesh colour, size and shape.

The original name of kiwifruit was 'Chinese gooseberry' and it was a name in common usage in New Zealand and elsewhere until it has been exported to the United States in 1959 . The idea to rename the fruit 'kiwifruit' is credited to Frieda Caplan, owner of Frieda's Finest Produce Specialitiea, which was among the first company to import the fruit into the United States. With its brown furry skin, which resembled New Zealand's iconic native bird the kiwi, Frieda suggested New Zealand growers to rename the fruit to get a better marketing response. Following this, the New Zealand fruit marketer Turners & Growers adopted this name and since then the name kiwifruit has achieved general acceptance across commercial, scientific and technical fields (Ferguson & Bollard, 1990). The name kiwifruit is now often used for all species within the genus *Actinidia.*

*Actinidia* species were introduced to Europe, the U.S.A. and New Zealand in the late 19$^{th}$ and early 20$^{th}$ century (Ferguson & Bollard, 1990). New Zealand was largely responsible for the initial development and commercial growing of kiwifruit. In 1904, Isabel Fraser, returned from her travel in China, introduced the first kiwifruit seeds to New Zealand upon which the New Zealand kiwifruit industry was built. By 1910 the plants raised by a friend, Alexander Allison, produced the first fruit outside China. *Actinidia deliciosa* cv. Hayward was selected around 1925 and kiwifruit production started in New Zealand with the first commercial orchards established in 1930s and the first commercial exports of fruit of *A. deliciosa* started in 1953 (Ferguson & Bollard, 1990).

Until 2000 *A. deliciosa* cv. Hayward was the backbone of the global kiwifruit industry and almost all the international trade in kiwifruit was of this sole cultivar. When facing overproduction in the early 1990s, the New Zealand industry innovated and assessed the commercial potential of another species, *Actinidia chinensis* (Ferguson & Huang, 2007).

Domestication and breeding of firstly A*ctinidia deliciosa and* more recently, *A. chinensis,* from wild germplasm resulted in a lot of commercially cultivated varieties worldwide distributed. The most important steps in the development of kiwifruit as a world commercial crop are summarized in Table 1.1. Until very recently, world trade in kiwifruit had developed from the stage with one predominant green-fleshed cultivar to one commercially important yellow-fleshed cultivar with other emerging yellow-

fleshed cultivars and finally to the initial commercialization of red-centered yellow-fleshed cultivars. However, all this has changed with the arrival in most countries producing kiwifruit of the disease caused by *Pseudomonas syringae* pv *actinidiae* (Ferguson, 2013).

**Table 1.1:** Important steps in the domestication and commercialization of kiwifruit.

| | |
|---|---|
| **1899** | First plants of *A. deliciosa* grown outside China |
| **1904** | Plants of *A. deliciosa* first sold in England |
| **1904** | Seed of *A. deliciosa* arrive in New Zealand |
| **1910** | First fruit of *A. deliciosa* produced outside China |
| **ca. 1930** | Establishment of the first commercial *A. deliciosa* orchard in New Zealand |
| **ca. 1930** | Selection of the *A. deliciosa* cultivar 'Hayward' |
| **Late 1930** | First commercial orchard of 'Hayward' |
| **1952** | First commercial coolstorage of *A.deliciosa* kiwifruit |
| **1953** | First commercial exports of *A. deliciosa* kiwifruit from New Zealand |
| **1959** | Invention of the name kiwifruit |
| **1961** | First cultivation of *A. chinensis* |
| **1965** | First commercial crop of *A.deliciosa* kiwifruit outside New Zealand |
| **1975** | Only 'Hayward' *A. deliciosa* fruit accepted for export from New Zealand |
| **1982** | First fruit of *A. chinensis* known to be produced outside China |
| **1997** | Start of branding of kiwifruit in international markets |
| **1998** | Start of commercial production of red-centered *A. chinensis* kiwifruit in China |
| **2000** | Launch of yellow-fleshed *A. chinensis* kiwifruit in international market |

## The genus *Actinidia*

The first specimens were collected in Nepal in 1821 by botanist Nathaniel Wallich but the genus was established in the 1836 by Lindley that recognized the specimens as belonging to a genus that could be distinguished by its climbing habitat and the unusual radiating arrangement of the styles. Until then the taxonomy of *Actinidia* has remained equivocal. Lindley placed the new genus in the Dilleniaceae, giving the name *Actinidia* for the stylar arrangement (from the Greek, *actis,* ray) and described the first species *A. callosa*, but only in 1899 Van Tieghem established the family *Actinidiaceae* containing *Actinidia* and *Saurauia* genus. The new family designated was distinguished by the presence of raphides, the versatile anthers, the carpels being accreted into a unilocular fruit, the nature of the embryo and the structure of ovules (Ferguson, 1984). Year by year, additional species and varieties were discovered and published, including *A. chinensis,* published by Planchon in 1847, *A. eriantha* and *A. strigosa* published by

Bentham in 1860. Early classification of the genus however, was extremely confusing; many *Actinidia* species were initially placed in different genera. *Actinidia latifolia* was first placed in the genus *Heptaca* (a doubtful genus in Tiliaceae) by Bentham in 1849, then in the genus *Kadsura* (Schisandraceae) by Miquel in 1861. *Actinidia rufa*, *A. arguta* and *A. polygama* were first placed in the genus *Trochostigma* in 1843, then transferred to *Actinidia* genus several years later. *Actinidia kolomikta* was variously placed in *Prunus, Kalomikta* and *Trochostigma* genera before finally being identified as *Actinidia* by Maximowicz in 1859 (Hsieh *et al.*, 2011). A comprehensive studied on the taxonomy of the genus was carried out by Dunn that first revised the genus *Actinidia* in 1911, establishing four sections, *Leiocarpae*, *Ampulliferae, Maculatae* and *Vestitae* based on the degree of pubescence, shape of ovary and presence or absence of lenticels on the fruit surface. These sections may be keyed as follow:

Fruit without spots:

- Sect. *Ampulliferae*: Ovary bottle-shaped;
- Sect. *Leiocarpae:* Ovary cylindric;

Fruit with spots:

- Sect. *Maculatae:* Leaves glabrous;
- Sect. *Vestiae:* Leaves shaggy or woolly;

In this first revision 24 species have been recognized and almost 40 varieties or forms worldwide (Dunn, 1911). The second major revision of *Actinidia* genus was carried out by Li in 1952 that included the section *Ampulliferae* into the section *Leiocarpae,* in order to eliminate the ambiguous character of ovary shape because such species as *A. tetramera* Maxim have the ovary intermediate shape. He further split the section *Vestitae* into *Stellatae* and *Strigosae* sections emphasizing the structure of leaf hairs. These sections may be keyed as follow:

- Sect. *Leiocarpae:* Fruit without spots;

Leaves without stellate hairs:

- Sect. *Maculatae:* Branch and petiole glabrous;
- Sect. *Strigosae:* Branch and petiole strigose;

Leaves with stellate hairs:

- Sect. *Stellatae*;

In the second revision were described 36 species and over 50 varieties or forms (Li, 1952). The scheme proposed by Li was adopted also by Liang which completed a

revision of Chinese *Actinidia* in 1984. Liang described many new taxa and listed 51 species as occurring in China, but estimated that there are 54 species within the genus (Liang, 1984).

The classification of the genus *Actinidia* is difficult and the taxonomy of some taxa is still confusing.

In the most recent revision within the genus *Actinidia* was achieved by Li in 2007 describing 20 varieties and about 55 species, most of them worldwide (Li & Soejarto, 2007) and all of this have morphological features in common: the climbing growth habitat, the structure of the fruit, the characteristic radiating arrangement of styles female flowers and the dioecy (Ferguson, 2013).

The classification of some taxa still needs further study. The species of *Actinidia* are highly variable in their vegetative structures, as well as in their flowers and fruits, which is the main reason for the difficulty in the classification of the genus (Li, 1952).

Morphologically, species of the genus *Actinidia* may be clearly separated into two major groups: the first group, which includes *Leocarpae*, has a glabrous ovary and the fruit has no spots; the second group, which includes *Maculatae*, *Strigosae* and *Stellatae*, has a hairy ovary and the fruit has spots (Li *et al.*, 2009).

All species in the genus *Actinidia* are seemingly dioecious, therefore there are female (fruiting) and male (pollenizer) plants (Schmid, 1978). Female plants have flowers with well-developed ovaries and styles as well as stamens. These flowers look as if they were hermaphroditc (perfect) but the pollen is not viable. After pollination, the ovules develop into seed and the ovaries into fruit: such flowers are functionally female (pistillate). Male plants have flowers with rudimentary ovaries which do not contain viable ovules; they cannot set seeds and the ovaries do not develop into fruits. Their stamens produce viable pollen and the flowers are functionally male (staminate) (Ferguson, 2013). Gender on *Actinidia* species appears to be controlled by an XX/XY system with the male plants having the Y chromosome. Two tightly linked genes are thought to determine gender: one stops pollen development in the female flowers and the other suppresses development of the ovary and ovules in male flowers. (McNeilage, 1997; Testolin *et al.* 1999). However the dioecy is not absolute, the flowers can be also bisexual able to perform the self-pollination and self-setting. Gender inconstancy has been observed in *A. arguta, A. chinensis* and *A.deliciosa* and

probably occurs in other *Actinidia* species (McNeilage, 1991). Male and female plants can differ in morphology with considerable variation in the size, shape and pubescence of leaves produced on different shoots at different times of the year even on the same plant and there can be transitional forms between taxa that overlap geographically (Dunn, 1911).

## *Actinidia species* in cultivation

Since *A. deliciosa* cv. Hayward (Figure 1.2 a) has been domesticated in New Zealand around 1930, it was considered the backbone of the global kiwifruit industry and has continued to perform extraordinarily well on the global market in terms of production and sales, it remains the dominant commercial kiwifruit cultivar. The Hayward variety arises in New Zealand from a number of competing varieties to become the choice of growers, with its ability to meet all the necessary characteristic needed for a commercially successful cultivar, including taste, storage and size qualities (Ward & Courtney, 2013). Until 2000 *A. deliciosa* 'Hayward' was the cultivar of choice and almost all the international trade in kiwifruit was of this sole cultivar.

When facing overproduction in the early 1990s, the New Zealand industry innovated and assessed the commercial potential of another species, (Ferguson & Huang, 2007). The new cultivar, developed in New Zealand in 1997 by HortResearch (now Plant & Food Research), entered on the international market in 2000 under the name ZESPRI® GOLD Kiwifruit (Figure 1.2 b), reflecting the peculiar golden-yellow fruit flesh. The introduction of ZESPRI® GOLD with its different look, color and taste showed the way in bringing new customers to the kiwifruit category. The most obvious difference between *A. chinensis* and *A. deliciosa* is hairiness of the fruit, the first one has smooth skinned fruits compared with *A. deliciosa,* colour (*A. chinensis* being usually yellow compared with the green fruit *A. deliciosa*), fruit flavour, flower size, shoot hairiness, geographic distribution, chromosome number and leaf shape (Ferguson & Bollard, 1990). The introduction of the yellow flesh cultivar *A. chinensis* 'Hort16A' changed the industry by offering a product that complemented, rather than competed, with 'Hayward' resulting in an increased consumption (Belrose Inc, 2012). Since 2000, most newly planted orchards in New Zealand have been *A. chinensis* 'Hort16A' and

represents about 26% of the New Zealand export of kiwifruit (Belrose Inc, 2012). After introduction of ZESPRI® GOLD, a range of new cultivars were commercialized in China and Japan, some of which also internationally. *A. chinensis* cv. Jintao or ENZAGold™, a yellow-fleshed cultivar selected in Wuhan, China, (Huang *et al.*, 2002), is widely planted in Italy (Ferguson & Huang, 2007). Recently, *A. chinensis* cultivar 'Hongyang' (Figure 1.2 c) selected in China, with a distinctive yellow-fleshed fruit and a brilliant red around the central core, is widely cultivated for the export market particularly in Japan (Wang *et al.*, 2003). To date, most cultivars have been selected from *A. chinensis* and *A. deliciosa*, however *A. arguta* are now commercially cultivated in USA, Chile and New Zealand (Ferguson & Huang, 2007). The fruits of *A. arguta* are smaller, smooth-skinned, with a rich and sweet flavour (Figure 1.2 d) (Williams *et al.*, 2003). Despite the breeding efforts 'Hayward' is still the predominant fruit traded internationally, with an estimate of 90 to 95 % of all kiwifruit market (Ferguson & Seal, 2008).

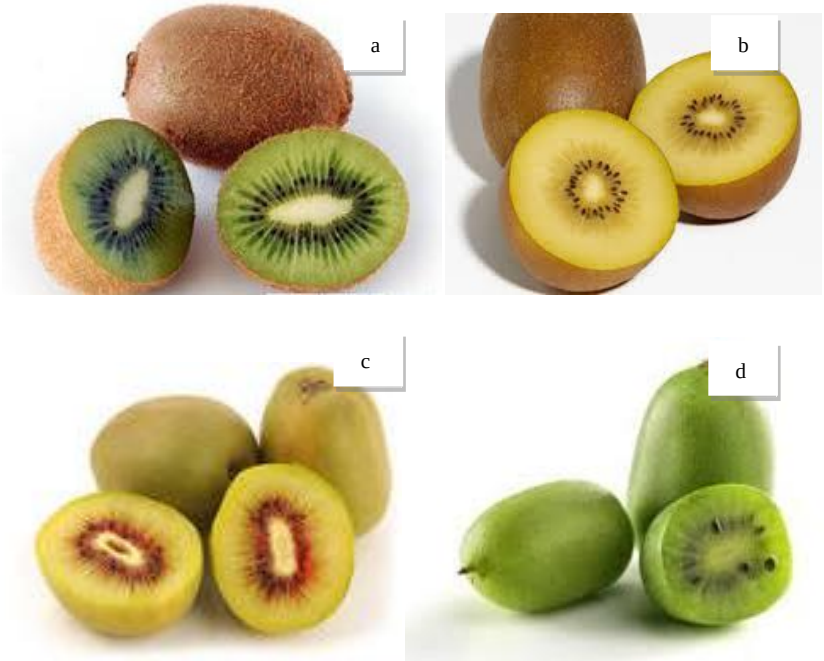


**Fig 1.2:** Commercially produced kiwifruit: (a) *A. deliciosa,* green fleshed kiwifruit, (b) *A. chinensis* 'Hort16A', gold yellow fleshed fruit, (c) *A. chinensis* cv 'Hongyang', yellow-fleshed fruit and brilliant red around the central core, (d) *A. arguta,* berry sized green kiwifruit.

## Global kiwifruit industry

*Actinidia* species were introduced to Europe, the U.S.A and New Zealand in the late 19th and early 20th century (Ferguson & Bollard, 1990). Commercial kiwifruit growing areas have expanded rapidly and consistently since records began in 1970, with exponential growth in the 1980, static production in 1990 and steady growth over the past decade. The growth of the industry has varied significantly in short bursts and the long-term growth path has continued upward with global production doubling over the past 20 years, furthermore it is predicted that this will continue as new plantings reach full maturity in key production countries such as China and Chile (Figure 1.3) (Ward & Courtney, 2013).

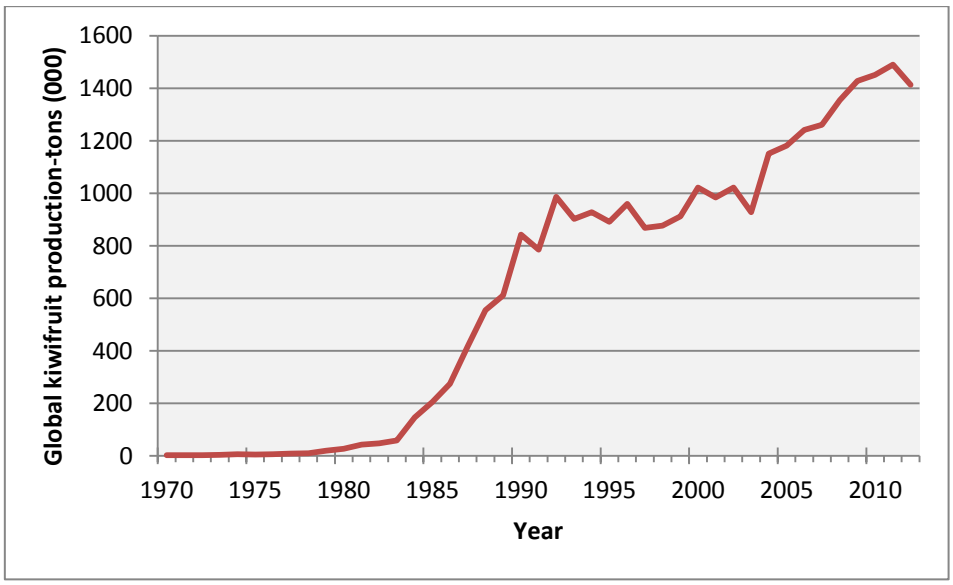


**Fig 1.3:** Global production of kiwifruit from 1970 to 2012. *Source: FAOSTAT (2014)*

The international kiwifruit production is concentrated in relatively few countries. The top four countries are, China, Italy, New Zealand and Chile that collectively produce more than 80% of the world's kiwifruit crop; the top ten producing countries represent more than 96% of the world supply. Total production in 2009-2012 was 1,862,000 tonnes (Table 1.2) (Belrose Inc., 2012).

**Table 1.2:** World kiwifruit production: Top-ten producing countries 2009-2012

| Rank | Country | Production (tons) (Average) |
|---|---|---|
| **1** | China | 480,000 |
| **2** | Italy | 450,000 |
| **3** | New Zealand | 372,833 |
| **4** | Chile | 230,333 |
| **5** | Greece | 83,167 |
| **6** | France | 71,851 |
| **7** | Japan | 33,300 |
| **8** | Iran (Islamic Republic of) | 31,532 |
| **9** | United States of America | 27,391 |
| **10** | Spain | 18,125 |

The growth in global kiwifruit production corresponded to an increase of the kiwifruit planted area. The Food & Agriculture Organisation of the United Nations statistical department (FAOSTAT, 2014) estimates in 1970 that there were < 1,000 ha of kiwifruit planted in the world outside of China. In 2010, the area of kiwifruit planted globally (including China) was estimated by O'Rourke to be over 160,000 ha (Figure 1.4) (Ward & Courtney, 2013).

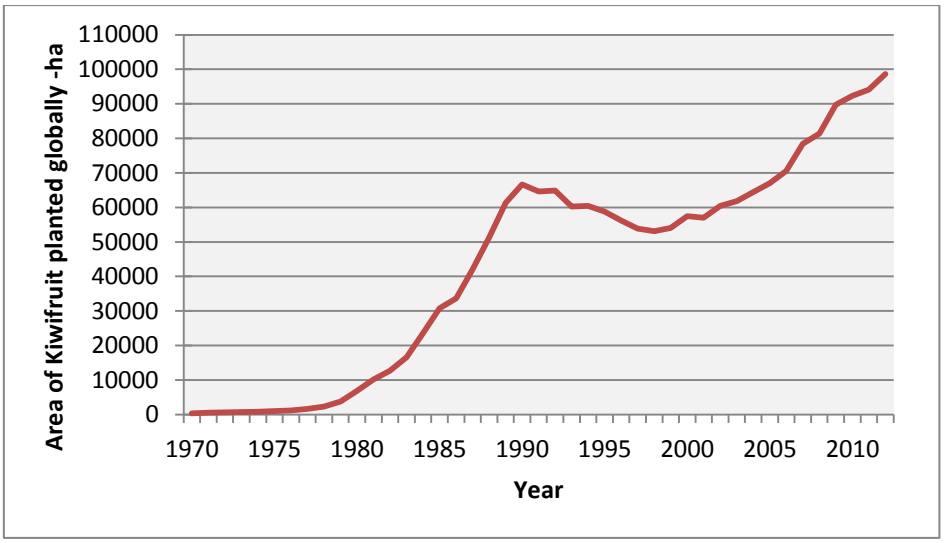


**Fig 1.4:** Area of kiwifruit planted globally from 1970 to 2012. *Source: FAOSTAT (2014)*

The growth of the global kiwifruit industry has not been simply as result of using more land, but the global average yields per hectare have also increased significantly, from an estimated 5,000 in 1970 to almost 15,000 kg $ha^{-1}$ in 2012 (FAOSTAT, 2014).

Resulting of the increased the global volume of kiwifruit produced was also the increasing of the global exports volume of kiwifruit. According to O'Rourke (2012), around two-thirds of global kiwifruit production is exported, with Italy, New Zealand and Chile as the world's leading exporters of kiwifruit. These countries accounted for about 90% of all exporters of kiwifruit in 2010. China is the largest producer but the production has been almost totally consumed in its domestic market with exports accounting for only 0.2 % of the global trade in kiwifruit (Table 1.3) (Ward & Courtney, 2013).

**Table1.3:** Major exporters: Share of global trade (volume) in fresh kiwifruit 2007-2010 (%).
*Source: O'Rourke (2012)*

| Exporter | 2007 (%) | 2008(%) | 2009(%) | 2010(%) |
|---|---|---|---|---|
| Italy | 31.1 | 28.6 | 32.7 | 31.0 |
| New Zealand | 32.5 | 35.1 | 30.7 | 30.8 |
| Chile | 14.9 | 14.9 | 15.5 | 15.2 |
| Greece | 3.4 | 3.5 | 4.7 | 6.2 |
| France | 2.7 | 2.4 | 1.9 | 2.2 |
| Spain | 1 | 0.9 | 1 | 1 |
| United States | 0.7 | 0.6 | 0.5 | 0.7 |
| Portugal | 0.3 | 0.1 | 0.3 | 0.5 |
| China | 0.3 | 0.2 | 0.1 | 0.2 |
| All other | 12.9 | 13.7 | 12.6 | 12.2 |
| **Total** | **100.0** | **100.0** | **100.0** | **100.0** |

On the world kiwifruit review in 2012, the value of the global kiwifruit industry in 2012 was estimated to be around US$ 1.9 billion (O'Rourke, 2012). Until the global financial crisis in 2008, the global value of exports of fresh kiwifruit had been increasing every year since 1999, with significant growth rates between 2002 and 2008 (Figure 1.5) (FAOSTAT, 2014). During this period, consequently to this strong growth, the area of kiwifruit planted increased around 22,000 ha (FAOSTAT, 2014).

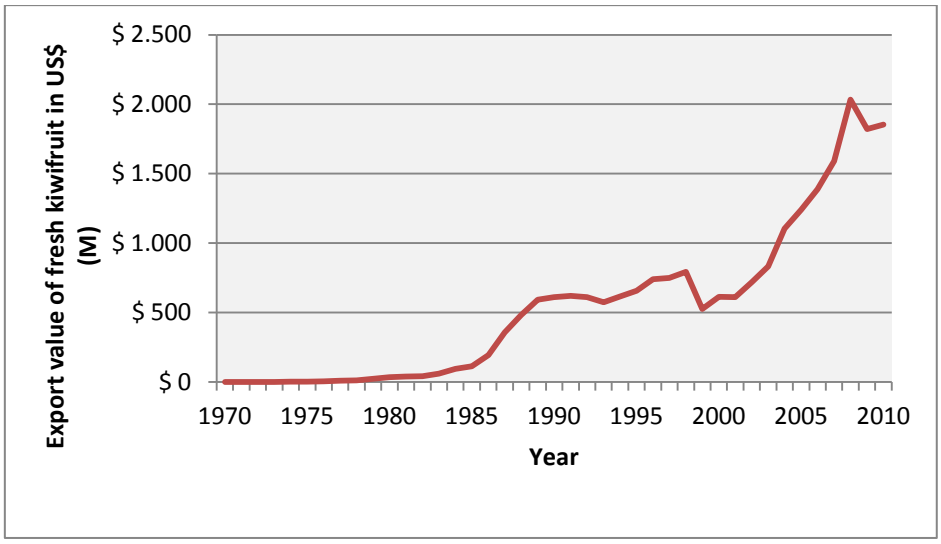


**Fig 1.5:** World value of exports of fresh kiwifruit. *Source: FAOSTAT (2014)*

Despite the significant growth in the global volume of kiwifruit over the past 30 years, the fruit has remained very much a niche product, accounting for less than a quarter of a percent of global fruit production. Global kiwifruit production represents about 0.22% of total production for major fruit crops, with the majority of kiwifruit consumed as fresh fruit (Table 1.4) (O'Rourke, 2012). World production has remained dominated by the traditional categories of citrus, apples, bananas and grapes. Inevitably, consumption of kiwifruit is highest in the countries that produce it. New Zealand is the largest consumer of kiwifruit with an estimated annual consumption level to be more than 5 kg of fruit *per capita*, while Spain, Chile, Italy and Portugal all have annual consumption level between 2 and 3 kg of kiwifruit *per capita* (O'Rourke, 2012).

**Table1.4:** World share of production for major fruit groups. *Source: O'Rourke (2012)*

| Fruit category | 1983-1985 (% of total) | 1996-1998 (% of total) | 2008-2010 (% of total) | 2008-2010 versus 1996-1998 (% change) |
|---|---|---|---|---|
| Apple | 12.81 | 13.28 | 11.49 | - 1.79 |
| Other deciduous | 9.68 | 9.66 | 10.87 | + 1.21 |
| Total deciduous | 22.48 | 22.94 | 22.36 | - 0.58 |
| Total grapes | 20.35 | 13.59 | 11.29 | - 2.30 |
| Oranges | 13.84 | 14.70 | 11.48 | - 3.22 |
| Other citrus | 7.60 | 7.25 | 8.96 | + 1.71 |
| Total citrus | 21.45 | 21.95 | 20.44 | - 1.51 |
| Bananas | 12.94 | 13.89 | 16.86 | + 2.97 |
| Other tropical | 21.49 | 21.21 | 22.45 | + 1.24 |
| Total tropical | 34.43 | 35.10 | 39.31 | + 4.21 |
| Other fresh fruit | 0.0 | 5.09 | 5.19 | + 0.10 |
| Total berries | 1.18 | 1.12 | 1.19 | + 0.07 |
| Kiwifruit | 0.11 | 0.21 | 0.22 | + 0.01 |
| ***Total fruit*** | 100.00 | 100.00 | 100.00 | n.a. |

## Production and marketing in Italy

The first kiwifruit orchard were established in Italy more than 40 years ago, around 1967 and since then the commercial kiwifruit growing areas have expanded rapidly and consistently. The Italian kiwifruit industry is important both nationally and internationally. It makes up only a small part of Italian horticulture in general, but is profitable and expanding. Italy is one of two world's biggest kiwifruit exporter together to New Zealand (Testolin & Ferguson, 2009).

Kiwifruits have been grown in Italy for more than 70 years, but for much of that time were simply as horticultural curiosities. Experimental plantings of *A. deliciosa* were established in 1966-67 at Lake Maggiore and over the following 2 or 3 years, small demonstration planting were established in other part of Italy using a mixture of plants sourced from the United Kingdom, including the recognized New Zealand cultivars such as 'Hayward'. One of the first attempts at commercial-scale kiwifruit plantings in Latina in 1971, probably using plants from New Zealand, failed because the plants died from heat stress after being grown within individual plastic domes under the misapprehension that kiwifruit were tropical plants (Testolin & Ferguson, 2009). By 1973, 40-50 ha have been planted and the success of these orchards encouraged establishment of further kiwifruit orchards in Piedmont, Lazio, Veneto and,

subsequently, in Emilia Romagna. Five years later, in 1978, between 600 and 800 ha of kiwifruit have been planted in Italy, of which perhaps 250 ha were productive or potentially productive (Zuccherelli & Zuccherelli 1981 ). The area in kiwifruit from 1984 to 2013 increased more than 10-fold, with an initial rapid increase to 18,000 ha from 1984 to 1990, followed by a decade during which the area remained essentially unchanged or even fell slightly and then a slowly increased until 2013 (Figure 1.6).

Production data follow much the same trend, an initial rapid increase from 1984 to 1990, then a plateau with wide fluctuations in production from year to year, followed by slow increase from 1999 (Figure 1.7). At least some kiwifruit plantings have been attempted in all the different regions of Italy but orchards have mainly consolidated only in four regions: Lazio, Piedmont, Emilia Romagna and Veneto (Table 1.5), with a smaller plantings in Abruzzo, Basilicata, Calabria, Campania, Lombardy and Apulia. Over the last 20 years, the biggest increases in area and production have been in south Lazio and Piedmont. Kiwifruit have become less important in regions such as Lombardy, because of frequent problems with frost and Apulia, because of damage caused by salt-laden winds and because economic conditions now favor the growing of alternative crops (Testolin & Ferguson, 2009).

Total area of kiwifruit planted in Italy from 2007 to 2012 is approximately the same but the tonnes produced fell slightly (Table 1.6)

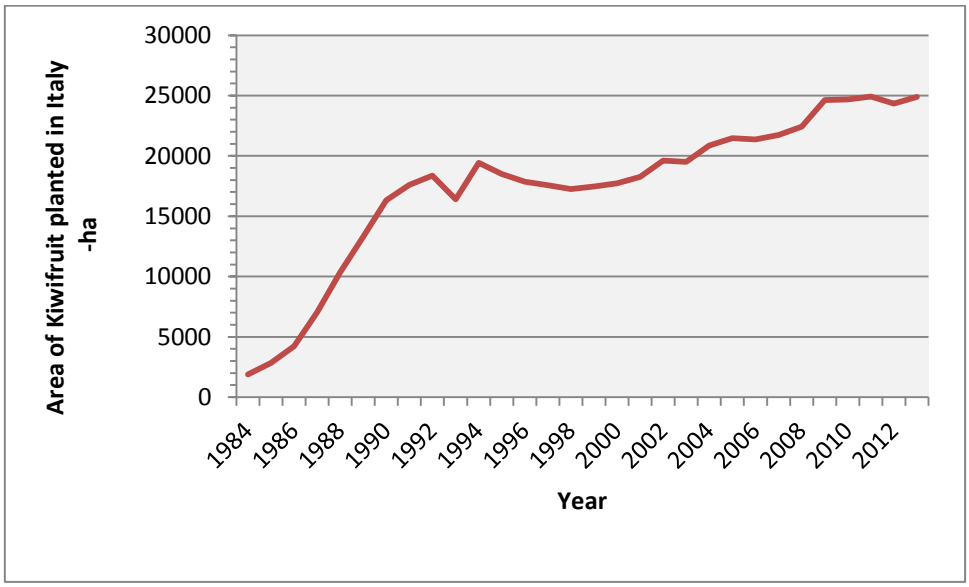


**Fig 1.6:** Area of kiwifruit planted in Italy from 1984 to 2013. *Source: FAOSTAT (2014)*

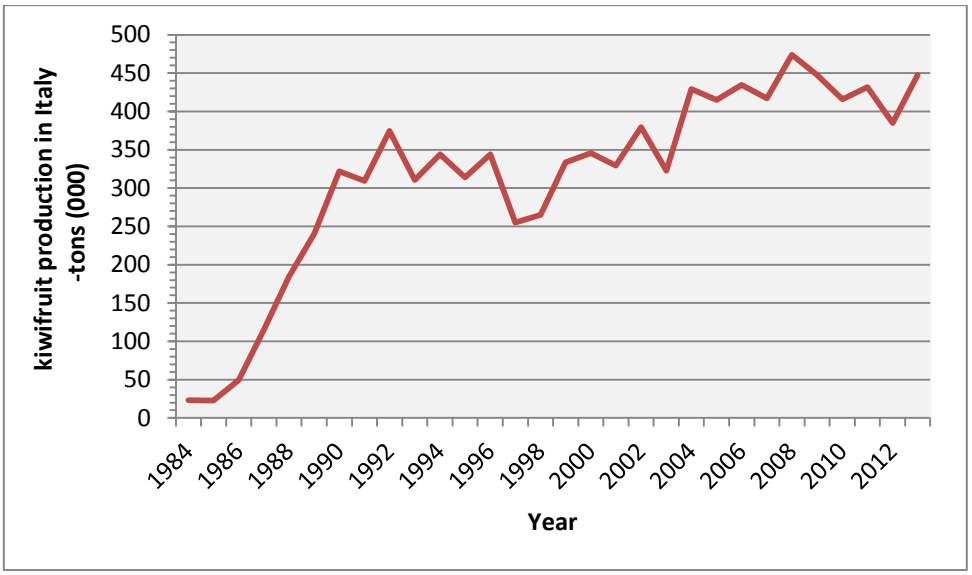


**Fig 1.7:** Production of kiwifruit from 1984 to 2013. *Source: FAOSTAT (2014)*

**Table 1.5:** Italian regions kiwifruit production: Top-four producing regions 2013. *Source: CSO (Centro Servizi Ortofrutticoli, Ferrara) (2014)*

| Region | % Total Area | Area (ha) |
|---|---|---|
| Lazio | 30 | 7,350 |
| Piedmont | 20 | 5,000 |
| Emilia Romagna | 16 | 4,000 |
| Veneto | 15 | 3,700 |

**Table 1.6:** Area of kiwifruit (ha) planted and production in Italy from 2007 to 2013. *Source: CSO (Centro Servizi Ortofrutticoli, Ferrara)(2012) and FAOSTAT (2014)*

| | 2007 | 2008 | 2009 | 2010 | 2011 | 2012 | 2013 |
|---|---|---|---|---|---|---|---|
| **Area of kiwifruit planted (ha)** | 26,834 | 27,275 | 27,619 | 28,300 | 28,058 | 26,893 | 24,891 |
| **Production of kiwifruit (t)** | 417,151 | 477,100 | 475,790 | 410,522 | 471,929 | 376,327 | 447,560 |

The initial development of the Italian kiwifruit industry was based on cultivars of *A. deliciosa* originating in New Zealand. 'Abbott', ' Bruno', 'Monty' and 'Hayward' were all planted experimentally (Ferguson & Bollard, 1990) but by 1983 over 70% of plantings were 'Hayward' and this reliance continued to increase for many years so that eventually it was essentially the sole fruiting cultivar grown. Only recently other cultivars of *A. deliciosa* and *A. chinensis* have started to produce commercial quantities

of fruit (Table 1.7) but 'Hayward' still accounts for an overwhelming 92% of female kiwifruit plantings in Italy (Testolin & Ferguson, 2009).

**Table 1.7:** Tons Commercial kiwifruit cultivars in Italy from 2007 to 2013 (t)
*Source: CSO (Centro Servizi Ortofrutticoli, Ferrara)(2014)*

| | 2007 | 2008 | 2009 | 2010 | 2011 | 2012 | p2013 |
|---|---|---|---|---|---|---|---|
| ***A. deliciosa*** | | | | | | | |
| **G3** | | | | | | | 100 (t) |
| **Summer** | 2.700 (t) | 3.400 (t) | 3400 (t) | 3770 (t) | 3800 (t) | 3.650 (t) | 2.700 (t) |
| ***A. chinensis*** | | | | | | | |
| **HORT 16A** | (*) | 14.200 (t) | 15.000 (t) | 10.000 (t) | 3.600 (t) | 4.000 (t) | 2.500 (t) |
| **Jingold** | 380 (t) | 2.570 (t) | 4.625 (t) | 5.782 (t) | 5.294 (t) | 6.345 (t) | 5.865 (t) |

* data not available

Even though Italy is one of the biggest producers of kiwifruit, the industry is still relatively small when considered in the context of the total Italian fruit production: kiwifruit account for around 2% of the total area of fruit crops and almost 3% of the total fruit production by weight (Source : FAOSTAT 2014) (Testolin & Ferguson, 2009).

## Diseases in *Actinidia* spp.

Commercial kiwifruit growing areas have expanded rapidly and consistently since records began in 1970 and plants has been considered to be relatively diseases free until recently. Just some fungal disease was divulged previously, such as *Armillaria novae-zelandii* identified in New Zealand in 1992 (Horner, 1992), *Phomopsis* sp. in Greece in 2009 (Elena, 2009), *Cadophora melinii* identified in Italy in 2008 (Prodi *et al*, 2008) and verticillum wilt of gold kiwifruit in Chile (Auger *et al.*, 2009).

Recently the phytosanitary situation of kiwifruit is changed radically with the detection of a virulent strain of *Pseudomonas syringae* pv*. actinidiae* (PSA), first in Italy and after in New Zealand (Ferrante & Scortichini, 2010; Everett *et al.*, 2011). During spring and autumn 2008 and winter 2008–9, severe outbreaks of bacterial canker were observed on *A. chinensis* (yellow kiwifruit) cvs Hort 16A and Jin Tao cultivated in central Italy (Latina province). The main typical symptoms were the oozing of reddish exudates

along the main trunk and branches, reddening of the lenticels under the epidermis, leaf spots sometimes surrounded by a chlorotic halo, leaf wilting, twig dieback and plant wilting (Ferrante &Scortichini, 2010). This pathogen was isolated for the first time in the same area from *A. deliciosa* cv. Hayward in 1992 (Scortichini, 1994) but from that time until 2008 it caused only sporadic damage (i.e. leaf spotting, twig dieback), always towards *A. deliciosa*. Severe damage and/or epidemics were never observed. The epidemic affecting 'Hort16A' in Italy was caused by a strain that appeared to be more virulent than a strain reported in 1994 (Ferrante & Scortichini, 2010). Since 2008 PSA was spread quickly and with considerable aggression, affecting plantations in the provinces of Latina and Rome (Lazio), in Emilia-Romagna especially in the provinces of Ravenna and Forlì, in Veneto, in Piedmont and, more recently, in Calabria. The epidemic in Italy has caused severe vine losses, with removal of entire orchards as a consequence (Figure 1.8) (Scortichini *et al.,* 2012; FAOSTAT, 2014).

Symptoms resembling those caused by PSA were first observed on *A. chinensis* in Te Puke, Bay of Plenty, New Zealand in November 2010 (Everett *et al*, 2011). Since then the disease has spread widely throughout the Bay of Plenty and also in other part of New Zealand that produce kiwifruit. The number of PSA positive orchards is now over 2,700, with 12,009 hectares (87%) on orchard where PSA has been identified (Figure 1.9) (Data from Kiwifruit Vine Health PSA Statistics Report, February 2015)

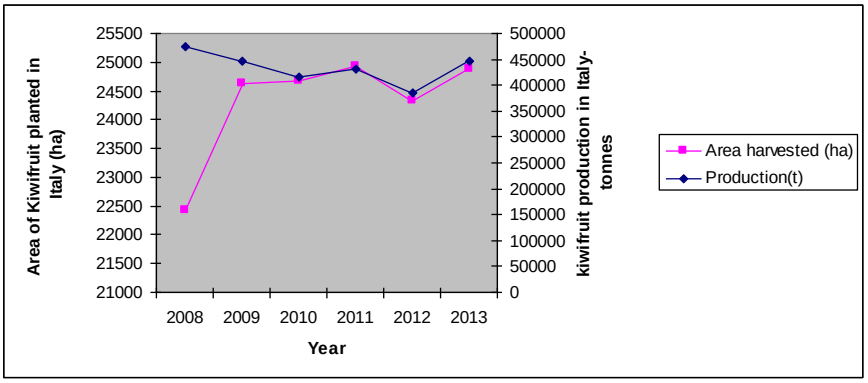


**Fig 1.8**: Area of kiwifruit planted in Italy and Production of kiwifruit from 2008-2013 during the PSA epidemic. *Source: FAOSTAT (2014)*

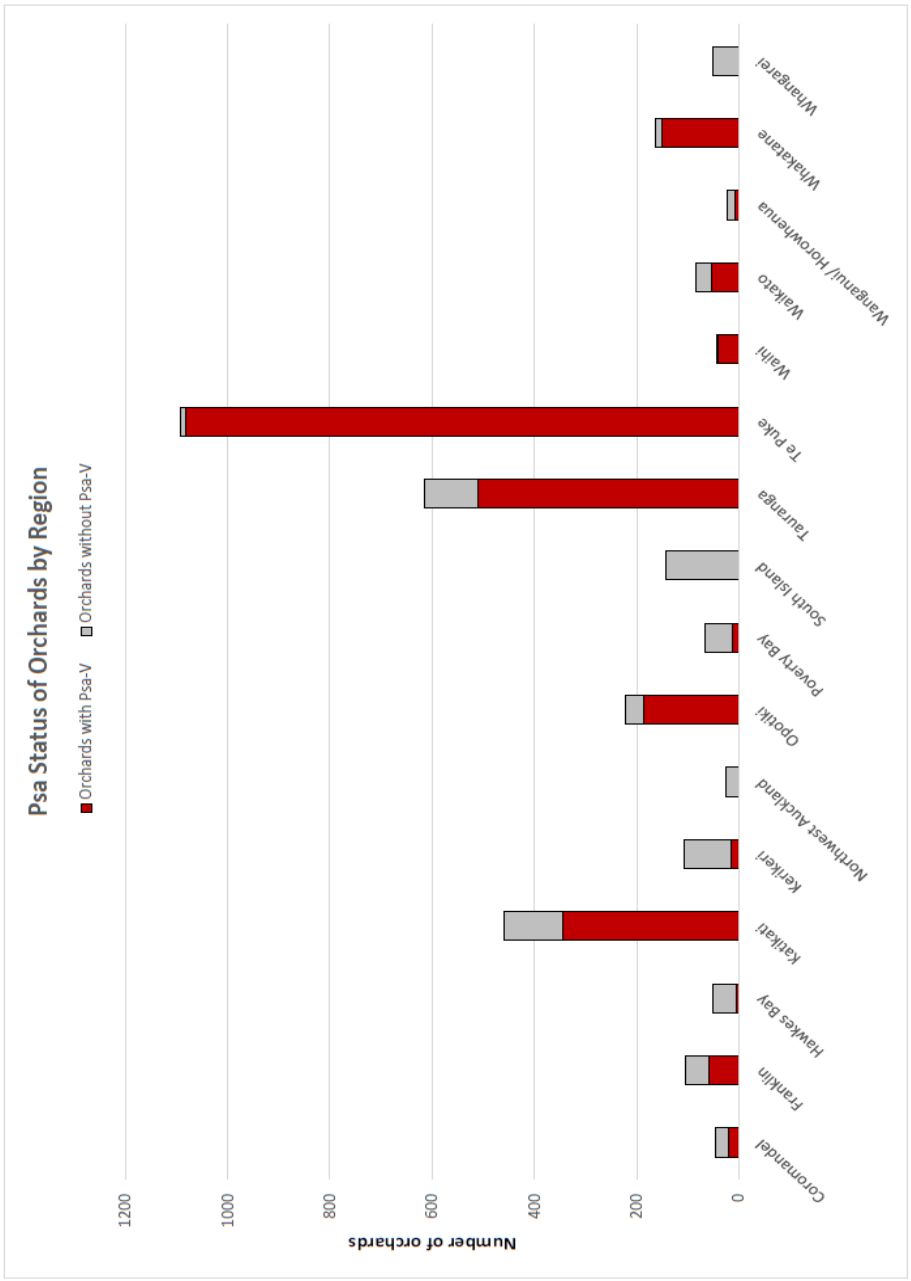


**Fig 1.9**: PSA status of orchard by region in New Zealand. *Source: KWH Kiwifruit Vine Health, 2015)*

## The aims of my study

In Italy, as well as in many other countries, the kiwifruit crop has been considered to be relatively disease-free and then no certification system for this species has been developed to regulate the import of propagation plant material in the European Union. The detection of a virulent strain of *Pseudomonas syringae* pv. *actinidiae* (PSA) has dictated the need to reorganize the certification system for this species in order to regulate import and exchanges of propagation plant material.

In attempting to address this issue, we also filled the lack of scientific knowledge on viruses infecting kiwifruit. Therefore, in order to study viral agents of this species, a project has been developed at the University of Bologna (Italy) in collaboration with the University of Auckland (New Zealand).

The aims of my PhD thesis were:

- to investigate and characterize the viruses that can infect kiwifruit plants in order to define the virological framework of the culture in Italy;
- to determine the best methods of investigation that can identify viral agents known or not yet known to infect *Actinidia* spp.*;*
- to characterize a strain of *Cucumber mosaic virus* detected in both, *A. chinensis* and *A. deliciosa*;
- to characterize a strain of *Pelargonium zonate spot virus* detected in *A. chinensis;*
- to characterize two novel viruses, a new putative *Closterovirus* and a new putative *Totivirus*, detected by the next generation sequencing approach;

The investigations of the viruses that can infect kiwifruit plants was carried out using biological, serological and molecular techniques. The characterization of the viral isolates has been also completed with the employ of the next generation sequencing (NGS) method, which has been useful also for identification of infections caused by multiple viruses.

*The results obtained during my PhD studies are proposed in chapters 2 to 5. Chapter 2 includes a review regarding kiwifruit viruses. The manuscript, published on the Journal of Plant Pathology is an "Invited Review" that the editors of the journal asked to our teams (New Zealand and Italy). The published paper is also included as Annex A.*

*Chapters 3, 4 and 5 report first identification and characterization of viral agents that infect kiwifruits in Italy. Each chapter is presented as a Project Paper then in the form that will be submitted to international scientific journals.*

*This format may generate some repetitions (in particular regarding Introduction, Materials and Methods and References sections) but, in my opinion, allow a better presentation of results obtained and, for sure, will speed up their publication.*

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# Chapter 2:

# Virus infecting *Actinidia* spp.

## Viruses of kiwifruit

The first clue of a kiwifruit infecting virus comes from New Zealand quarantine records in 1983. Gary Wood, from the Department of Industrial and Scientific Research (DSIR, New Zealand), documented local lesions observed on *Chenopodium quinoa* after sap inoculation of kiwifruit imported from China and held in quarantine. The infected kiwifruit plants were either destroyed or died during thermotherapy (G. Wood, personal comunication). In the 1980s, as Italy was becoming an important kiwifruit producer with the second greatest area planted worldwide, there were no records of viruses infecting the crop.

Caciagli and Lovisolo (1987) surveyed commercial orchards for potential viral diseases and collected samples from 100 symptomless *A. deliciosa* and one plant of *A. deliciosa* that showed chlorotic mottling. The extracts from these plants were mechanically inoculated into four herbaceous indicators (*C. quinoa*, *C. amaranticolor*, *Nicotiana glutinosa* and *N. clevelandii*). None of the 404 inoculated indicator plants displayed symptoms. Additionally, the authors challenged young *A. deliciosa* plants with 17 common viruses from Italy, including *Alfalfa mosaic virus* (AMV) and *Cucumber mosaic virus* (CMV). Only three viruses, *Tobacco necrosis virus* (TNV), *Tobacco rattle virus* (TRV) and CMV, induced symptoms on the inoculated leaves of the kiwifruit and only CMV moved systemically. The authors concluded that kiwifruit may be resistant to virus infections. A few years later, during a survey in the Fujian Province in China, Lin and Gao (1995) identified one plant showing a “mosaic disease” attributed to an unidentified virus. Nitta and Ogasawara (1997) reported evidence of a graft-transmissible agent causing viruslike symptoms. Using cuttings from *Actinidia polygama* plants collected in the mountains of Hiroshima Prefecture (Japan) as rootstocks, they observed chlorotic spots and rings on the eight different *A. deliciosa* varieties used as male scions. In neither case the causal agent was identified. In 2003, *Apple stem grooving virus* (ASGV) was identified in a kiwifruit import from China held in New Zealand quarantine (Clover *et al.*, 2003). This first virus identified in kiwifruit was detected by leaf symptoms, transmission electron microscopy (TEM) and mechanical transmission to herbaceous indicators and

identified by DAS-ELISA, RT-PCR and sequencing of amplicons. Other kiwifruit from the same consignment were subsequently studied further and new viruses were identified.

To assess the potential risk from viruses to kiwifruit, it is important to document which viruses are present in both breeding material and commercial crops and as far as possible determine where they originated from and how do they spread. In some cases viruses have been moved internationally with germplasm, while in other cases the viruses may have infected kiwifruit locally, from other plant species, following the introduction of the crop. Although China is the origin of *Actinidia* until relatively recently most of the breeding and selection of commercial cultivars was conducted in NZ involving movement of *Actinidia* germplasm from China to New Zealand over several decades. Subsequently there has been movement of commercial cultivars from New Zealand to several countries around the world, including Chile, Italy and back to China. In addition there has also been recent movement of germplasm and commercial varieties from China to Italy. Examination of kiwifruit germplasm and field crops in New Zealand and Italy, between 2002 and 2013 has identified a total of 13 different viruses, representing a wide taxonomic range (Table 2.1). However, many of the source plants were infected by multiple viruses which has made it difficult to attribute symptoms to individual viruses.

Recently two new detection were described outside of New Zealand and Italy, first one in China where *Actinidia virus A* and *Actinidia virus B* were detected on *A. chinensis* (Zheng *et al.,* 2014) and the second one in India with the characterization of *Apple stem grooving virus* infecting *A.deliciosa* (Bhardwaj *et al.,* 2014).

**Table 2.1**. Viruses detected naturally infecting *Actinidia* spp.
* data refers to the virus species in general, not specifically the *Actinidia* isolate
(?) = presumed mode of transmission based on properties of related viruses

| Virus name | Genus | Distribution | | Host range* | Mode of transmission * | Field spread in Actinidia | First report in kivifruit |
|---|---|---|---|---|---|---|---|
| | | In Actinidia | All hosts | | | | |
| *Actinidia virus A* | *Vitivirus* | NZ, Italy, China, Japan (?) | no known alternative host | *Actinidia* | mealybugs (?) | No | Blouin *et al.* (2012) |
| *Actinidia virus B* | *Vitivirus* | NZ, Italy, China, Japan (?) | no known alternative host | *Actinidia* | mealybugs (?) | No | |
| *Alfalfa mosaic* | *Alfamovirus* | NZ | worldwide | very wide | aphids, seed | No | Pearson *et al.* (2011) |
| Actinidia virus X | *Potexvirus* | NZ | unknown | *Actinidia* | mechanical | No | |
| *Apple stem grooving virus* | *Capillovirus* | China, NZ | worldwide | apple, pear citrus, Lilium | seed | No | Clover *et al.* (2003) |
| *Cherry leafroll virus* | *Nepovirus* | NZ | worldwide | wide | flower thrips seed, pollen | Yes | Woo *et al.* (2012b) |
| *Citrus leaf blotch virus* | *Citrivirus* | China, NZ | Europe, USA, Australia, Japan, NZ | *Actinidia*, citrus | seed | No | Pearson *et al.* (2011) |
| *Cucumber mosaic virus* | *Cucumovirus* | NZ, Italy | worldwide | very wide | aphids, seed | No | Pearson *et al.* (2009) |
| *Cucumber necrosis virus* | *Tombusvirus* | China | Canada, China, NZ | wide | ***Olpidium radicale*** | No | Lebas *et al.* (unpublished) |
| *Pelagonium zonate spot virus* | *Anulavirus* | Italy | Italy | *Pelargonium*, tomato, artichoke | seed, pollen | Yes | Biccheri *et al.* (2012) |
| *Ribgrass mosaic virus* | *Tobamovirus* | NZ | worldwide | wide | mechanical | No | Chavan *et al.* (2009) |
| *Turnip vein clearing virus* | *Tobamovirus* | NZ | worldwide | wide | mechanical | No | |
| *Closterovirus (unidentified)* | *?* | NZ, Italy | no known alternative host | *Actinidia* | aphid (?) | No | Biccheri *et al* (unpublished) |

To date, the viruses discovered in kiwifruit can be divided in three groups. The first group comprises AMV, ASGV, CMV, *Cucumber necrosis virus* (CNV), *Ribgrass mosaic virus* (RMV), *Turnip vein clearing virus* (TVCV) and a novel potexvirus, tentatively named Actinidia virus X (AVX). These viruses are mostly ubiquitous/ cosmopolitan and, so far, do not show a detrimental effect on commercial kiwifruit. Most of these viruses are distributed worldwide over a large host range and have been detected in alternative hosts neighboring kiwifruit orchards. The second group comprises the putatively kiwifruit specific viruses that, to date, are only known to have this single host or are likely to have a very limited host range. In this group we have identified two vitiviruses, *Actinidia virus A* (AcVA) and *Actinidia virus B* (AcVB) and a citrivirus closely related to *Citrus leaf blotch virus* (CLBV). There is also evidence of a novel virus from the family *Closteroviridae,* Actinidia latent virus (AcLV). The third and most concerning group includes two viruses that have very recently been detected in kiwifruit. *Cherry leaf roll virus* (CLRV) in New Zealand and *Pelargonium zonate spot virus* (PZSV) in Italy both cause severe damage to the commercial crop. In addition, the viruses listed in Table 2.1 represent a range of different modes of transmission with varying consequences for disease spread and control. From an epidemiological perspective the known viruses can be sub-divided into those with no known natural vectors, those with aphid vectors, those with presumed mealybug vectors and those transmitted by seed and/or pollen. Almost 10 years since the first publication of kiwifruit virology, we describe now the 13 viruses detected in kiwifruit to date. This represents the first review of kiwifruit viruses, including images of symptoms (Figure. 2.1), a summary table of each virus (Table 2.1) and a summary of diagnostic tools including primer sequences and amplification conditions (Table 2.2).

## Non-Specialist viruses

### *Alfalfa mosaic virus* and *Cucumber mosaic virus*

AMV and CMV are two viruses infecting a very broad host range, with over 1200 plant host species in over 100 families for CMV (Douine *et al.,* 1979) and 300 species in 22 plant families for AMV (Hull, 1969). The addition of *Actinidia* species to their host range is not unexpected. Because of the damage CMV causes on some economically important crops, it was included in the "Top 10 plant viruses" in a recent molecular pathology review (Scholthof *et al*., 2011). Both viruses belong to the family *Bromoviridae* and are efficiently vectored by a number of aphid species. They are also transmitted by seed and are easily transmissible mechanically. AMV is the type member of the genus *Alfamovirus* and has four bacilliform type particles (Fauquet *et al*., 2005).

CMV is the type member of the genus *Cucumovirus* and has icosahedral particles. AMV was one of the first viruses detected and identified in kiwifruit in New Zealand (Pearson *et al.*, 2009). It was first detected in *Actinidia glaucophylla,* showing strong yellow mosaic patterns (Figure. 2.1A). Extracts from the chlorotic blotch easily transmitted the virus to a range of herbaceous indicator plants. In the same germplasm collection, AMV was also isolated from *Actinidia guilinensis* and *A. fortunatii* showing mottled and generally chlorotic leaves. In these hosts, the plants looked unthrifty and the virus symptoms were widespread in the block. The symptoms were observed in spring for four consecutive years. AMV and CMV have been found as a dual infection in both *A. glaucophylla* and *A. fortunatii and* CMV was also detected in a single symptomless infection of *A. glaucophylla.* AMV has only been detected once in *A. chinensis* in New Zealand. The plant showed a few leaves with very minor chlorosis and the symptoms could not be observed the following year. Inoculation of AMV to *A. chinensis* seedlings induced foliar symptoms on one or two leaves above the inoculated leaf, but newer leaves were symptomless.

**Table 2.2.** Diagnostic tools: reagents for ELISA when available and primers used and conditions for PCR assays.

| Virus Name | ELISA | PCR | | | | |
|---|---|---|---|---|---|---|
| | | Forward primer | Reverse-primer | Annealing (°C) | Amplicon size (bp) | Reference |
| AMV | Bioreba (Switzerland) Cat 140512-140522 Only reliable for symptomatic *Actinidia* tissue and herbaceous indicators | AMV for TGTCTCACTGATGACGTG | AMV rev CATACCTTGACCTTAATCCAC | 55 | 415 | Blouin *et al*., 2010 |
| CMV | Bioreba, Cat 160612 and 160622 | CMV-F CTTTCTCATGGATGCTTCTC<br><br>CMV nF (nested if required) ACTATTAACCACCCAACCT | CMV-R GCCGTAAGCTGGATGGAC<br><br>CMV nR (nested if required) TTTGAATGCGCGAAACAAG | 54 | 885<br><br>Nested: 172 | Felix and Clara, 2008 |
| PZSV | ADGEN Phytodiagnostics | PZSV2 F GATAAATTCAGAGCTCTCGG | PZSV2 R ATCTCTGCAGATTGTGTTCC | 55 | 997 | Biccheri *et al*, unpublished |
| AcVA | Not available | AcVA 1F ATGATGGGGTGTTCTATGGGTGGCT | AcV1R CTCATTCTCCAMCCRCARAAGAG | 55 | 269 | Blouin *et al*., 2012 |
| AcVB | Not available | AcVB1F AATTCGGACCACTCCTGAGGC | AcV1R CTCATTCTCCAMCCRCARAAGAG | 55 | 529 | Blouin *et al*., 2012 |
| AVX | Rabbit Polyclonal Antiserum Raised Against Purified virus (Plant & Food Research) | AVX-F (3963) AAGTCCGCAACACCTACCTG | AVX-R (4118) GGACAGACGATAGCAGCCTT | 58 | 175 | Cohen and Blouin, unpublished |
| CNV | DSMZ (Germany), antisera AS-0130 | PCR1 Gral. Tombusvirus F1 AAGGGTAAGGATGGTGAGGA<br><br>CuNV-F791 (nested) CCTCGCAGAAGACCTTATGC | PCR1 Gral. Tombusvirus R1 TTTGGTAGGTTGTGGAGTGC<br><br>CuNV-R1002 (nested) GCCGACTCCTCCACTCCA | PCR1 55<br><br>Nested-PCR 60 | PCR1 587<br><br>Nested-PCR 215 | PCR1 Harris *et al.,* 2007<br><br>Nested-PCR Lebas *et al,* unpublished |
| CLRV | Bioreba, Cat 160612 and 150812 | CLRV-F TGGCGACCGTGTAACGGCA | CLRV-R GTCGGAAAGATTACGTAAAAGG | 55 | 416 | Werner *et al*., 1997 |
| ASGV | Bioreba, Cat 150822 and 150812 | CTLV-AP CCTGAATTGAAAACCTTTGCTGCCACTT | CTLV-AM TAGAAAAACCACACTAACCCGGAAATGC | 60 | 456 | Ito *et al*., 2002 |
| Actinidia citrivirus | Dweet mottle antiserum Antiserum USDA253, (courtesy of Dr Richard Lee) | CLBV 1F AGCCATAGTTGAACCATTCCTC | CLBV 5R GCAGATCATTCACCACATGC | 58 | 425 | Chavan*, et al* manuscript in preparation |
| RMV and TVCV | Rabbit polyclonal antiserum raised against purified TMV (Auckland University) | AT2F AGACAGCAATTCTCAAACTTGT | AT 4R CGGTCGCATCATCAACAC | 55 | 223 | Chavan *et al*, unpublished |

CMV has been detected in Italy on one *A. chinensis* plant with pale mottling of the leaves (See chapter 3). AMV and CMV can be detected by RT-PCR in *Actinidia* spp. (Table 2.2). DAS-ELISA can also be used for both viruses but AMV can only be detected in symptomatic tissues. Both viruses are readily transmissible to a range of herbaceous indicators including *N. benthamiana*, *N. clevelandii*, *N. glutinosa* and *N. occidentalis*. These two viruses are similar in terms of their abundance in the surrounding weeds and also by sharing the same vectors. Both are present worldwide and are likely to infect *Actinidia* spp. causing some concerns for the non-commercial species (*A. glaucophylla, A. guilinensis* and *A. fortunatii*). Fortunately, the viruses do not appear to have a detrimental effect on either *A. chinensis* or *A. deliciosa*. Their impact on these important crops is therefore negligible.

***Ribgrass mosaic virus* and *Turnip vein clearing virus***

RMV and TVCV are two closely related species in subgroup 3 of the genus *Tobamovirus*, family *Virgaviridae*. Both viruses have 300 nm rod-shaped particles with positive sense, single-stranded RNA (ssRNA) (Adams *et al*., 2009). RMV was first reported from *Plantago* (Holmes, 1941) and has been variously referred as Holmes ribgrass virus, *Tobacco mosaic virus*-ribgrass strain, Crucifer TMV and TMV Wasabi (Gibbs, 1999). It has been reported from at least 67 different species belonging to 15 diverse dicotyledonous and monocotyledonous families (Chavan *et al*., 2012). Symptoms include systemic chlorotic mottling, ring-like markings, chlorotic streaks along the veins and twisting of the petioles in *Plantago* species, vein clearing in turnip (Lartey *et al*., 1993), necrotic mosaic in tobacco and internal browning of tomato fruit (Oshima and Harrison, 1975). Tobamoviruses have no known natural vectors but the particles are stable and readily mechanically transmitted. They can also be carried and transmitted from the surface of seeds (Gibbs, 1977). RMV was first detected in *A. deliciosa* and *A. chinensis* held in post-entry quarantine in New Zealand (Chavan *et al*., 2009) and the complete sequences of the isolates from *A. chinensis* (GenBank accession No.

GQ401366.1) and *A. deliciosa* (GQ401365.1) were subsequently published (Chavan *et al*., 2012). RMV and TVCV were first reported in New Zealand from *Plantago* spp. (Cohen *et al.,* 2012). Subsequent studies have identified both viruses in *A. chinensis* in New Zealand and TVCV has been identified in samples of dried leaf material of *A. chinensis* from both China and Italy (Cohen *et al.,* unpublished information). Both viruses were amplified by the primers designed to detect RMV (Chavan *et al*., 2012) and can only be distinguished by sequencing of the amplicons. Symptoms on *A. chinensis* include chlorosis of leaf veins and adjacent tissue during spring and chlorotic mottles, mosaics and ringspots during summer. Symptoms on *A. deliciosa* include chlorotic mottling or mosaic during spring and ringspots during summer months (Chavan *et al*., 2009). Some of the symptoms resemble those previously described in *Actinidia* infected with ASGV (Clover *et al*., 2003) and subsequent investigation has established that most of the plants were co-infected with other viruses (Chavan *et al*., unpublished information). Symptoms on mechanically inoculated indicators include local chlorotic lesions in *C. amaranticolor* and *C. quinoa,* systemic mosaic and distortion in *N. benthamiana,* systemic necrotic ringspots and chlorotic vein banding and dark green blistering and distortion in *N. clevelandii*, local necrotic lesions and systemic mottle in *N. glutinosa* and *N. occidentalis and* mild systemic mottle in *Phaseolus vulgaris* (Chavan *et al*., 2009), but some of these symptoms may be caused by co-infecting viruses. For routine diagnosis, RMV and/or TVCV can be detected in *Actinidia* leaf samples by conventional RTPCR (Table 2.2). ELISA, using a rabbit polyclonal antiserum raised against purified TMV (M. Pearson, The University of Auckland), detected *Actinidia* isolates of RMV in herbaceous indicators but failed to detect the virus in infected *A. chinensis* and *A. deliciosa* plants (Chavan *et al*., 2009). There are no known arthropod vectors of tobamoviruses but they can survive in sap for prolonged periods (Oshima and Harrison, 1975). Tobamoviruses are highly infectious and readily spread by contact between infected and healthy plants or via machinery and human handling (Gibbs, 1977). Consequently, similar treatments to those recommended to prevent the spread of TMV, such as seed sterilisation using hypochlorite, should be used to prevent virus on seed coats from infecting seedlings during

nursery operations (Cohen *et al*., unpublished information). Overall, RMV and TVCV do not appear to cause significant damage to commercial kiwifruit orchards.

***Apple stem grooving virus***

ASGV is the type member of the genus *Capillovirus*, family *Betaflexiviridae*. Its genome consists of a positive-sense ssRNA of 6,496 nucleotides (excluding the polyA-tail) enveloped in a flexuous, filamentous particle of 620-700x12 nm. Citrus tatter leaf virus (CTLV) is regarded as an isolate of ASGV, being indistinguishable from it biologically, serologically and in genome organization. The main crop hosts are apple, European pear, Japanese pear, Japanese apricot, citrus and lilies and experimentally it infects more than 40 species in 17 plant families. It is probably found wherever apples are grown and natural spread has also been reported in citrus in China and Japan. Some *Lilium* ASGV strains can infect *Citrus* and a *Pyrus* isolate infects *Citrus* (Yoshikawa, 2000). The kiwifruit ASGV isolate from *A. chinensis* (AF522459) (Clover *et al*., 2003) has an identical genomic organization to strains from *Citrus*, *Malus* and *Lilium*, with a high degree of identity to *Citrus* (D16681), *Malus* (D14995) and *Lilium* (AB004063) isolates across the 32-terminal half (2,901 nt) of the genome. The coat protein and movement protein genes share a nucleotide identity of >95% with other strains of ASGV. The morphological, epidemiological, serological and molecular characteristics of the virus from *A. chinensis* are indistinguishable from those of ASGV from other hosts (Clover *et al.*, 2003). ASGV in kiwifruit was first detected in *A. chinensis* budwood from Shaanxi province, (China), grafted onto healthy rootstocks of *A. chinensis* cv. Hort16A and grown in post-entry quarantine in New Zealand. The original source of the plants, within China, is not known. Infected plants developed interveinal mottling, chlorotic mosaics and ringspots (Clover *et al.,* 2003). However, these plants were subsequently found to be co-infected with RMV and vitiviruses (R.R. Chavan, unpublished information). ASGV is often latent in commercial *Malus* and *Citrus* although it can cause graft union necrosis, tree decline and death in some apple (Yanase, 1983) and citrus (Broadbent *et al*., 1994) rootstock/scion combinations. It is unknown whether ASGV results in

significant yield losses in *A. chinensis* as it was detected in plants detained in post-entry quarantine under greenhouse conditions and observed for only a limited period of time (Clover *et al*., 2003). Some surveys for ASGV in *A. chinensis* have been carried out in New Zealand and ASGV was detected in extracts from some plants using RT-PCR and immunocapture RT-PCR (ICRT-PCR). Sequencing of amplicons confirmed the presence of ASGV, but repeated extractions from the same plants gave variable results, indicating that the virus was unevenly distributed in the plants. Attempts to isolate ASGV from orchard plants by inoculation to herbaceous indicator plants have never been successful (Cohen *et al*., unpublished information).

ASGV is transmissible by grafting and mechanical inoculation to herbaceous plants. Vectors and natural means of field transmission are unknown for isolates from *Actinidia*, *Malus* or *Citrus* (Yoshikawa, 2000; Clover *et al*., 2003). ASGV is seed-transmitted in *Lilium longiflorum* and *C. quinoa* (Inouye *et al*., 1979) but it is unknown whether the *Actinidia* isolates are seed-transmissible. The *Actinidia* isolate was graft-transmitted to *A. deliciosa* and produced the same symptoms as in the original host. It was also mechanically transmissible to a number of herbaceous hosts (Clover *et al.*, 2003). The symptoms observed on *C. quinoa*, *Phaseolus vulgaris* and *Vigna unguiculata* are very similar to those described for isolates from other hosts (Inouye *et al*., 1979; Zhang *et al*., 1988; Yoshikawa, 2000). For diagnostic purposes ASGV was successfully detected in infected indicator plants and directly from *Actinidia* samples by conventional RT-PCR using the primers (ML-F and ML-R, Table 2.2) of Ito *et al.* (2002). ASGV was also detected by ELISA, using ASGV antisera raised against apple strains of ASGV (Table 2.2) and ICRT-PCR. Both protocols were reliable but the ICRT-PCR was 50 times more sensitive than ELISA (Clover *et al*., 2003). Because the ASGV is thought to be transmitted in the field only by grafting, planting virus-free plants is the best means of controlling the virus. ASGV does not represent a threat to kiwifruit production.

***Cucumber necrosis virus***

CNV (genus *Tombusvirus*, family *Tombusviridae*) is an isometric virus of 31 nm diameter containing ssRNA (Dias, 1972). CNV was first described in 1959 on cucumber plants from Canada which appeared stunted with severe foliar symptoms. The virus is transmitted in soil by zoospores of the fungus *Olpidium radicale* [syn. *O. bornovanus*, *O. cucurbitacearum*; (Dias, 1970a, 1970b)] but not through seeds (McKeen, 1959). CNV can be mechanically transmitted to a wide host range including plants belonging to the families Amaranthaceae, Asteraceae, Chenopodiaceae, Cucurbitaceae, Fabaceae and Solanaceae (Dias, 1972). However, to date, the virus has only been found naturally to infect cucumbers (*Cucumis sativus*) in Canada (McKeen, 1959), lettuce (*Lactuca sativa*) and tomato (*Solanum lycopersicum*) in the USA (Obermeier *et al*., 2001) and kiwifruit (*Actinidia* spp.) in China, Italy and New Zealand (Lebas *et al*., unpublished information). In 2009, *A. arguta* and *A. deliciosa* plants were bought from a commercial garden centre in Auckland (New Zealand) to be used as healthy controls for PCR. Both plants were found to be infected with CNV when tested by ICRT nested-PCR (Table 2.2). The 215 bp sequences obtained from both species were identical (KC478972, KC478973) and had 99% nucleotide identity with CNV isolates from Canada (M25270) and New Zealand (DQ663769). Subsequent testing of imported Chinese *A. deliciosa* (KC478971) and Italian *A. deliciosa* plants confirmed the presence of CNV in this material (B.S.M. Lebas, unpublished information). *Actinidia arguta* and *A. deliciosa* plants were propagated in a local nursery that provides plants to commercial garden centres all around New Zealand, so CNV is likely to be widely distributed within the country. CNV causes necrotic spots, severe leaf distortion and stunting on greenhouse cucumber plants (McKeen, 1959). It elicits localized leaf necrosis on lettuce and was found in mixed infection with Lettuce necrotic stunt virus (LNSV, tenative species in the genus *Tombusvirus*) on tomato with leaf chlorosis and internal fruit necrosis in the USA (Obermeier *et al*., 2001). No symptoms were observed on the two infected *Actinidia* plants from New Zealand or on the imported material from China and Italy. In addition, CNV was only detected by ICRT nested-PCR, suggesting it was present at a very low titre in all the *Actinidia*

spp. plants tested. Therefore, it is likely that CNV is not a major pathogen of kiwifruit. Although CNV is detected in an increasing number of hosts, it has not been reported to cause any significant economic damage since the first report in 1959 (McKeen, 1959). CNV may have been present in New Zealand for some time. However, it has not been reported on any other crop species, although the vector *O. radicale* infects cucumber, tomato and beans (Pennycook, 1989). The impact of CNV on the kiwifruit production is unknown but is likely to be negligible.

**Actinidia virus X**

AVX is a novel putative potexvirus isolated on herbaceous indicator plants from three *A. chinensis* plants. The virus has flexuous particles of about 485 nm long and 12-13 nm width. Its sequence

(KC568202) shows the typical organisation of a potexvirus with five ORFs. ORF1 (nt 26-4825) encodes the putative replicase of 1,599 aa with a calculated mass of 180 kDa. It contains the methyltransferase domain at the N-terminal, the NTPase/helicase domain in the central region and the RNA-dependant RNA-polymerase domain in the C-terminal region (Martelli *et al.*, 2007).

ORF1 is followed by a short intergenic region of 52 nt and the triple gene block (TGB) formed by three overlapping ORFs; ORF2 (nt 4,878-5,585), ORF3 (nt 5,554-5,916) and ORF4 (nt 5,753-6,022) have a calculated mass of 26, 13 and 10 kDa respectively. ORF5 (nt 6,041-6,784) codes for a 26 kDa coat protein. Phylogenetic analysis showed the virus clustered with a subgroup comprising *Narcissus mosaic virus* (NMV), *Asparagus virus*-3 (AV-3), *Malva mosaic virus X* (MaMV) and Scallion virus X (ScVX). The nucleotide identity on the full genome varied between 64 and 65% with these viruses and between 57 and 59% nt identity with *Alstroemeria virus X* (AlsVX), *Lettuce virus X* (LVX) and *Pepino mosaic virus* (PepMV). AVX was easily mechanically transmissible to

*N. benthamiana*, *N. clevelandii* and *N. occidentalis and* it induced systemic symptoms in *C. quinoa*. Two out of the three isolations of the virus were made from samples of symptomatic kiwifruit. In these two plants, a vitivirus was also

detected. The two symptomatic plants were destroyed after sample collection and resampling was not possible. The third detection was from a symptomless plant but re-isolation, RT-PCR and ELISA failed to re-detect the virus. It is possible that the virus is cryptic in kiwifruit in the same way that AlsVX is latent in *Alstroemeria* (Fuji *et al*., 2005). Kiwifruit may not be the preferred host of AVX. The virus is probably distributed unevenly in kiwifruit plants and may occur at low titre, as it was only isolated on three occasions out of many hundreds of inoculations over the past 7 years. After purification of AVX from *N. occidentalis*, an antiserum was prepared from rabbit. Its successful use in indirect ELISA (plate-trapped antigen ELISA) was demonstrated from infected herbaceous indicators and leaves of *A. chinensis* seedlings that had been inoculated with the virus. AVX was detected at high titre in inoculated leaves of *A. chinensis* seedlings, but its titre gradually declined in new leaves over several months (Pearson *et al*., 2011). Inoculated leaves on these seedlings showed veinal necrosis but no symptoms were observed on systemically infected leaves (D. Cohen and A.G. Blouin, unpublished information). AVX can also be detected by RT-PCR (Table 2.2). This virus has so far only been isolated from *Actinidia* spp on to *Nicotiana* spp and *C. quinoa* no further information is available on its host range and distribution. However, based on the absence of symptoms in systemically infected *A. chinensis* seedlings and the low incidence of detection, the impact of AVX is likely to be very low.

## Kiwifruit-Adapted viruses

### Actinidia citrivirus

The Actinidia citrivirus has a monopartite, linear, positive-sense, ssRNA genome of 8,782 nt (JN900477) and shares 74% nucleotide identity with CLBV (AJ318061). The genome organization is identical to that of CLBV, with three non-overlapping open reading frames and a 3’ terminus poly(A) tract. ORF1 (nt 72-6,035), the putative replicase polyprotein, includes methyltransferase, AlkB, OTu-like peptidase, papainlike protease, RNA helicase and RNA-dependent RNA polymerase domains, typical of a citrivirus (Martelli *et al.*, 2007). It codes for

1,987 aa and has a calculated mass of 230 kDa. ORF2 (nt 6,035-7,123) codes for a putative movement protein of 362 aa has a calculated mass of 40 kDa. An intergenic region of 55 nts follows ORF2 before the start codon of ORF3 (nt 7,124-7,178). ORF3 codes for a 40 kDa coat protein (358 aa).

The 5' and 3' UTRs are 71 and 526 nt long, respectively (Chavan *et al*., 2013). CLBV is the type and currently the only recognised member of the genus *Citrivirus*. The Actinidia citrivirus has been detected only in kiwifruit scionwood material imported from China (Chavan *et al*., 2013). In *A. chinensis* the virus is associated with a range of symptoms, including vein clearing and mild mottling on leaves and interveinal chlorosis during summer, although some infected accessions remained symptomless. All of the symptomatic kiwifruit plants infected with the Actinidia citrivirus were found to be coinfected, making it difficult to attribute the symptoms to one virus alone (Figure 2.1B shows leaf symptoms of a plant co-infected with Actinidia citrivirus, AcVA and AcVB). No attempt has been made to inoculate the Actinidia isolate to citrus, the only known natural host of CLBV. The Actinidia citrivirus is transmitted by grafting in *Actinidia*, similarly to CLBV (Vives *et al*., 2001). The Actinidia citrivirus and CLBV have both been mechanically transmitted to a range of common herbaceous indicator plants including *N. benthamiana*, *N. clevelandii, N. glutinosa* and *N. occidentalis;* the citrus isolate of CLBV gave symptomless infections (Vives *et al*., 2008; Guardo *et al*., 2009) whereas the *Actinidia* isolate produced distinctive symptoms on *N. glutinosa* (Figue 2.1C) (Chavan *et al.*, 2013). Although Actinidia citrivirus isolates can be detected by ELISA using an antiserum against Dweet mottle virus [= CLBV (Antiserum USDA253, courtesy of Dr. Richard Lee] (D. Cohen and A.G. Blouin, unpublished information) and by PCR using primers designed from the coat protein gene of CLBV (Table 2.2), the Actinidia citrivirus shows several distinct differences. First, the symptoms induced in *N. glutinosa* (Figure 2.1C). Second, all sequences of CLBV deposited in GenBank show very high similarity with one another, whereas the *Actinidia* citrivus isolates show considerable sequence variation. Third, phylogenetic analysis has shown that from the 3' end of ORF1 to the 3' untranslated region (UTR) (including all of ORF2 and ORF3) the citrus CLBV and the Actinidia citrivirus share 78% identity at the nt

level and > 90% identity at the aa level. However, the 5' and 3' UTRs, as well as the 5' end of ORF1, show divergence of about 30% at the nt level (Chavan *et al.,* 2013). Based on current International Committee on Taxonomy of Virus (ICTV) demarcation criteria for sequence similarity within the family *Betaflexiviridae*, i.e. less than 72% nt identity or 80% aa identity in the CP or the polymerase gene (Adams *et al.,* 2011), Actinidia citrivirus is borderline for classification as a new species. Since means of natural spread of the Actinidia citrivirus are unknown, control relies on the use of virusfree scionwood and rootstocks in combination with good hygiene to prevent the possibility of mechanical transmission via pruning. The impact of the virus is likely to be very low, mostly due to the lack of a vector.

***Actinidia virus A* and *Actinidia virus B***

The genus *Vitivirus* was named after *Vitis* sp., host of the reference species *Grapevine virus A* (GVA). *Vitis vitifera* also hosts four additional vitiviruses, i.e. *Grapevine virus B*, *Grapevine virus D*, *Grapevine virus E* and Grapevine virus F (Adams *et al*., 2011; Al Rwahnih *et al.,* 2012). Most vitiviruses naturally infect a single host; the other natural vitivirus hosts currently known are mint (*Mint virus 2,* MV2) and heracleum (*Heracleum latent virus,* HLV) (Adams *et al*., 2011). Two novel vitiviruses *Actinidia virus A* (AcVA) and *Actinidia virus B* (AcVB) were detected in kiwifruit by RT-PCR (Blouin *et al*., 2012). Both viruses have a monopartite, linear, positive-sense, ssRNA genome. AcVB genome was fully sequenced (JN427015) and is 7,488 nt long and 7,566 nt of AcVA were sequenced (JN427014) covering all the genome but the 5'UTR and the beginning of the ORF1. They share 64% nucleotide identity and each comprises five ORFs: ORF1 codes for the replication genes with a calculated mass of 195 kDa. Both sequences include conserved domain for a methyltransferase, an AlkB, a RNA helicase and a RNA-dependent RNA-polymerase in respective order from the amino terminus to the carboxyl terminus as described for the genus in Martelli *et al.* (2007); AcVA has a lysine-rich insert between motifs I and II of the methyltransferase that is not present in other vitiviruses, including AcVB. ORF2 codes for a putative protein of unknown function and has a calculated mass of 25

and 27 kDa for AcVA and AcVB respectively. This is the most divergent gene of the virus with only 16% aa identity between them and no homology to any protein from GenBank; ORF3 (nt 5,704-6,597 and 5,698-6,570) codes for a movement protein with a calculated mass of 33 and 32 kDa respectively and share 56% aa similarity. ORF4 (nt 6,515-7,111 and 6,488-7,084) codes for the coat protein of a calculated mass of 21 kDa for both viruses. This is the most conserved gene of the viruses and AcVA and AcVB share 75% aa in common and are less than 70% aa similar to the closest vitiviruses (GVB and HLV). ORF5 (nt 7,112-7,429 and 7,085-7,405) codes for a putative RNA binding (RNA silencing inhibitor) protein of a calculated mass of 12 kDa (Blouin *et al*., 2012). As a consequence of the historical movement of plant material, the grapevine-infecting vitiviruses have been reported in most grapevine-growing regions. Vitiviruses are not known to be seed-transmitted and AcVA and AcVB have only been detected in accessions that were imported to New Zealand as scions, or in scions that have been grafted on to an infected plant (Blouin *et al*., 2012). AcVA and AcVB have also been detected in two Chinese scionwood accessions growing in Italy (D. Cohen and A.G. Blouin, unpublished information). Inoculation of sap from symptomatic vines of *A. chinensis* induced symptoms on *N. occidentalis*. The coat protein was partially purified from herbaceous indicator plants and a few peptides common to GVB were identified by tandem mass spectrometry (Blouin *et al*., 2010). A survey of more material showed symptoms ranged from large ringspots, vein chlorosis and mottle to symptomless plants, but some of the infected plants could host more viruses (Fig. 2B showing symptoms from a mixed infection including AcVA, AcVB and the Actinidia citrivirus). AcVA and AcVB were transmitted by grafting to *A. deliciosa* but the infected plants remained mostly symptomless (Blouin *et al*., 2012).

## Disease-Inducing viruses

### *Cherry leaf roll virus*

CLRV is an established species within subgroup C of genus *Nepovirus*, family *Secoviridae* (Sanfaçon *et al*., 2012). CLRV has been reported to be present in North America, Chile, Peru, Europe, China, Japan, Australia and New Zealand (Woo *et al*., 2012a). In addition to its worldwide distribution, the virus also has a wide natural and experimental host range, infecting members of more than 36 plant families (Walkey *et al*., 1973; Rebenstorf *et al*., 2006). This includes a variety of wild and cultivated, herbaceous and woody plant species. Unlike most nepoviruses, CLRV does not appear to be transmitted by soil-inhabiting nematodes. However, the virus has been documented to be transmitted by seed, pollen, grafting and mechanical inoculation to herbaceous hosts (Woo *et al*., 2012a). CLRV has a bipartite genome of two positive-sense, ss-RNA molecules. Each RNA molecule is encapsidated separately in an isometric particle that is about 28 nm in diameter. Both RNA molecules are required for virus infection (Le Gall *et al*., 2005). RNA-1 and RNA-2 have structural organization typical of the genus and comprise 7,905 and 6,511 nt, respectively (Eastwell *et al*., 2012). CLRV was first described in sweet cherry in England (Posnette and Cropley, 1955). Subsequently, it was found to cause leaf rolling and plant death in cherry (Cropley, 1961) and a range of other plant species including elderberry, olive, raspberry, rhubarb, walnut and a number of other shrub, tree, weed and ornamental species (Büttner *et al*., 2011; Woo *et al*., 2012a). CLRV was isolated from a *A. chinensis* cv. Hort16A orchard in which vines were showing necrotic symptoms on leaves (Figure 2.1D), as well as cane die-back and bark cracking. Some of the fruit from the infected vines do not have the beak at the calyx end that is characteristic of the Hort16A cultivar (Figure 2.1E). Additionally, the fruit from infected vines are uneven in size and the crop yield is reduced. Extracts from symptomatic leaves inoculated to herbaceous indicators induced large necrotic lesions on *N. occidentalis* and ringspots on *N. tabacum*. The virus was identified by RT-PCR and sequencing. The sequences obtained from infected kiwifruit (JN371141) closely match those of an isolate from raspberry in New Zealand (Jones and Wood, 1978) and described as group C (Rebenstorf *et al*.,

2006). Detection in symptomatic material is also possible with DAS-ELISA (Table 2.2). CLRV was also detected in *Rumex* spp. (JN371148) directly below the infected vines using DAS-ELISA. All these characteristics make CLRV a potential threat for kiwifruit production and future studies are required to understand fully its ecology.

***Pelargonium zonate spot virus*** **(See chapter 4)**

PZSV is the type species and the single member of the *Anulavirus* genus within the *Bromoviridae* family (Bujarski *et al*., 2012). Amazon lily mild mottle virus, a new virus, isolated from an Amazon lily plant, has been recently described and proposed as new anulavirus species (Fuji *et al*., 2012). PZSV was described as *Tobacco streak virus* when first detected on tomato plants in southern Italy (Martelli and Cirulli, 1969) and later designated as PZSV when isolated from *Pelargonium zonale* (Quacquarelli and Gallitelli, 1979). This virus has been reported on tomato, pepper and weed species from Italy, Spain, France, the USA, Israel and Australia (Gallitelli, 1982; Luis-Arteaga and Cambra, 2000; Gebre-Selassie *et al*., 2002; Liu and Sears, 2007; Escriu *et al*., 2009; Lapidot *et al.,* 2010; Luo *et al*., 2010).

Recently, PZSV has been detected in several symptomatic kiwifruit plants (*A. chinensis* cv. Hort16A) in Italy, from two orchards located in the Emilia-Romagna region. Infected plants showed chlorotic and necrotic rings on leaves (Figure 2.1F) and depressed areas on the fruits (Biccheri *et al.,* 2012). PZSV can be detected directly from symptomatic kiwifruit tissues by ELISA, dot blot DNA hybridization and RT-PCR (Table 2.2). With regard to the symptoms in the commercial orchard, PZSV is an important pathogen to manage. Further study will assess its spread efficiency, which will determine the seriousness of the disease.

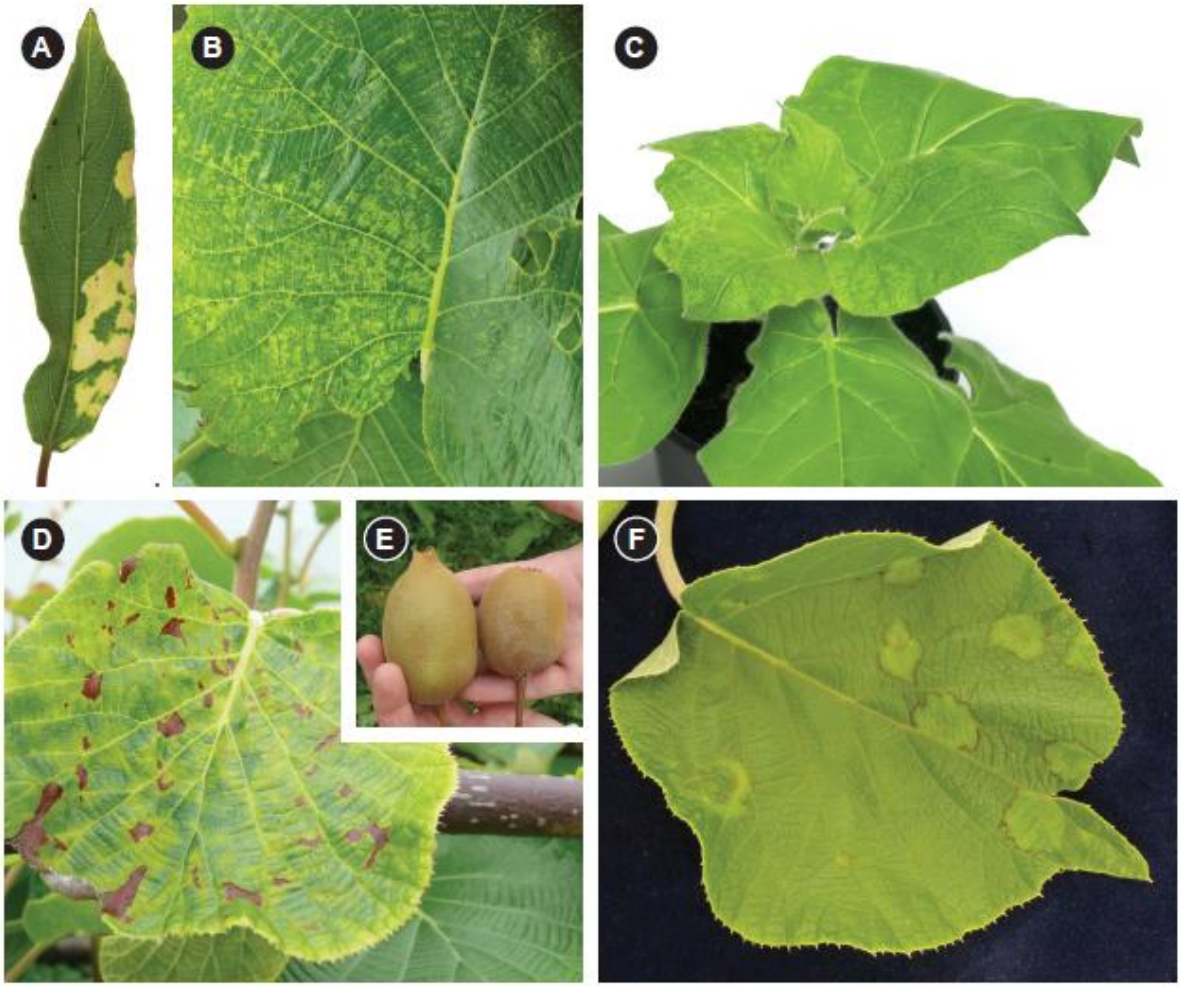


**Fig. 2.1** A. Symptomatic leaf of *Actinidia glaucophyla* infected with *Alfalfa mosaic virus*. B. Symptomatic leaf of *Actinidia chinensis* infected with *Actinidia virus A*, *Actinidia virus B* and Actinidia citrivirus. C. Symptomatic *Nicotiana glutinosa* infected with Actinidia citrivirus. D. Symptoms associated with *Cherry leaf roll virus* in *Actinidia chinensis* cv. Hort16A. Chlorosis developing into necrosis on a leaf. E. Symptoms associated with *Cherry leaf roll virus* (CLRV) in *Actinidia chinensis* cv. Hort16A, a regular fruit on the left with a beak at the calyx end characteristic of cv. Hort16A and fruit infected with CLRV on the right not showing the beak. F. Symptoms observed on leaves of *Actinidia chinensis* cv. Hort16A infected with *Pelargonium zonate spot virus*.

## Mechanically transmitted viruses with no known natural vectors

AVX (*Potexvirus*), ASGV (*Capillovirus*), RMV (*Tobamovirus*) and TVCV (*Tobamovirus*) have no known natural vectors but all are mechanically transmissible. Consequently, where possible, precautions should be taken to

avoid transmission on knives and secateurs during activities such as pruning. Tobamoviruses in particular are highly stable and infectious, have long survival times in plant sap and are easily spread by contact. ASGV is mechanically transmissible to herbaceous indicators, but has not been reported to be mechanically transmitted to apple in the field. However, ASGV from citrus (Citrus tatter leaf virus) has been experimentally transmitted from citron to citron by knife slashing (Roistacher *et al.,* 1980). Consequently the possibility of mechanical transmission of ASGV during pruning of kiwifruit cannot be excluded. In addition to being mechanically transmissible some isolates of ASGV are seed transmissible in *Lilium* spp. and *Chenopodium* spp and to a very limited extent in Eureka lemon (Tanner *et al*., 2011). Most potexviruses are easily mechanically transmissible but because the AVX was only detected from three *A. chinensis* plants in NZ, the impact of this virus is probably very low (Blouin *et al*., 2013).

## Viruses with aphid vectors

It is probable that these viruses, such as AMV and CMV, move into *Actinidia* from other hosts by aphids. Both viruses are typically difficult to control due to their wide host ranges and rapid spread by their aphid vectors. There is currently no evidence of severe effects on commercial crops. These viruses are easily mechanically transmitted and while precautions should be taken not to spread them by this means, attempted elimination of these viruses is unlikely to succeed because of the high probability of natural reinfection via aphids.

## Viruses with presumed mealy bug vectors

Two vitivirus species, AcVA and AcVB are potentially transmitted by mealy bugs. Grapevine vitiviruses are spread by mealybugs and scale insects. Vitiviruses are often detected as coinfections with a member from the family *Closteroviridae*. In grapevine, *Grapevine leafroll associated virus 1* (GLRaV-1 genus *Ampelovirus*) has been reported to be co-transmitted with the GVA (Hommay *et al.*, 2008). A

recent study using a donor plant with mixed infection of GVA and *Grapevine leafroll associated virus 3* (GLRaV-3) found that the majority of the receiving plants were infected with GLRaV-3 alone (24%) or both viruses (31%), while only 2% were infected with GVA alone and 43% were not infected (Blaisdell *et al*., 2012). In kiwifruit, no movement of the *Actinidia* vitiviruses has been observed in New Zealand other than by grafting and all the positive vines could be linked to an import of scionwood from China (Blouin *et al.,* 2012). Some of the plants had been imported for several decades. This lack of movement suggests that the virus is present either without its helper virus or without efficient vectors. All the novel vitiviruses were detected in co-infection then it is possible that both viruses share a common vector however, it is expected that they also exist as single infections in the wild. A virus that may potentially assist the natural transmission of Actinidia vitiviruses has been identified by next generation sequencing (NGS) in Italy in a plants also infected by AcVA and AcVB. This virus has the characteristics of a member of the family *Closteroviridae*.

The impact of vitiviruses on kiwifruit largely depends on their capacity to move and is therefore low in New Zealand. It is also too early to assess the impact of the novel putative closterovirus. Consequently, it should be possible to control the spread of AcVA and AcVB by planting propagation material that has been indexed for absence of these viruses.

## Viruses transmitted by seed and/or pollen

*Actinidia* viruses that are potentially seed transmissible include AMV, ASGV, CLBV, CLRV, CMV and PZSV. CLBV is also transmitted at a low percentage through seeds (Guerri *et al*., 2004), but so far there is no evidence that the Actinidia citrivirus can be mechanically transmitted by orchard operations and no seed transmission was observed within more than 300 *Actinidia* seedlings of an infected *A. chinensis* female parent; suggesting that if there is any seed transmission in kiwifruit, it would be at very low rate (D. Cohen and A.G. Blouin, unpublished). ASGV is seed transmissible in some hosts and because of the stability the tobamoviruses, RMV and TVCV, may be transmissible as surface

contaminants on seed, as is the case for other Tobamoviruses (Salamon and Kaszta, 2000; Seoun, 2001).

CLRV and PZSV are both viruses pollen transmitted in other hosts (Lapidot *et al.*, 2010; Card *et al.,* 2007) and consequently have the potential to spread rapidly within individual and between orchards, although pollen transmission in kiwifruit has not yet been proven. Within kiwifruit orchards, the virus seems to spread along the row, suggesting a possible mechanical spread by pruning/girdling equipment. CLRV has a worldwide distribution and wide host range, including many woody species and was found in a gold kiwifruit orchard (Blouin *et al*., 2013). PZSV is seed borne in some herbaceous species and is also transmitted via pollen in combination with thrips feeding (Vovlas *et al*., 1989).

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# Chapter 3:

# Project Paper:

## Characterization of *Cucumber mosaic virus* naturally infecting *Actinida chinensis* in Italy

# Characterization of *Cucumber mosaic virus* naturally infecting *Actinida chinensis* in Italy

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## Abstract

*Cucumber mosaic virus* (CMV) has a worldwide distribution and widest host range of any known plant virus. CMV causing systemic mosaic, necrotic local lesions and ringspot lesions has been isolated from kiwifruit (*A. chinensis*) plant leaves collected in the Emilia Romagna region, Italy. The determination of causal agent has been based on host range, symptom expression in the test plant species and morphological properties of the virus particles using transmission electron microscopy (TEM) and using specific oligonucleotide primers in reverse transcription-polymerase chain reaction (RT-PCR). Phylogenetic analyses show that CMV detected in kiwifruit belongs to CMV subgroup IA. To our knowledge this is the first characterization of CMV isolate infecting naturally actinidia in Italy.

## Introduction

Kiwifruit, originated from China, belongs to the genus *Actinidia* and is an important crop grown in temperate regions. The first commercial orchard were established around 1930 in New Zealand. China (480,000 tons) Italy (450,049 tons), New Zealand (372,833tons) and Chile (230,333tons) are the four world's largest producers (Belrose, 2012) and Italy and New Zealand are the two world's biggest kiwifruit exporters then the Italian kiwifruit industry is important both nationally and internationally (Testolin & Ferguson, 2009). In Italy, kiwifruit cultivation started in 1970 and rapidly increased its production and by 2007 the kiwifruit orchards occupied more than 26,000 ha (Testolin & Ferguson, 2009) mainly localized in few regions such as Lazio, Piedmont, Emilia Romagna and Veneto.

In many country, including Italy, the phytosanitary situation was almost unconsidered as only some fungal disease were identified, such a *Cadophora melinii* in Italy (Prodi *et al*., 2008). Kiwifruit crop has been therefore considered to be relatively disease free. After the increasing of the kiwifruit as a commercial crop in Italy, Caciagli and Lovisolo (1987) artificially infected actinidia plants by several of the most common viruses. Only the *Cucumber mosaic virus* (CMV) induced systemic symptoms so the authors concluded that *Actinidia* spp. may possess resistance to main viruses infection. The first definitive identification of a virus infecting kiwifruit plants was in 2003 with the detection of an *Apple stem grooving virus* (ASGV) strain in cultivars from China, held in quarantine in Auckland, New Zealand (Clover *et al.,* 2003). Subsequently more viruses have been detected. Examination of kiwifruit germplasm and field crops in Italy and New Zealand, between 2003 and 2013 has identified a total of 13 different viruses, representing a wide taxonomic range. The properties and detection methods for these viruses are described in details by Blouin *et al.* (2013). In this paper we also report the presence of CMV in *A*. *chinensis,* in Italy.

CMV, discovered almost 100 years ago in cucurbits, belongs to the genus *Cucumovirus* within the family *Bromoviridae.* The virus can infect a very broad hosts range, with over 1200 plant host species in over 100 families (Douine *et al.,* 1979) therefore the addition of *Actinidia* species to its host range is not unexpected. In recent times, CMV has caused severe epidemics in many crops and it was included in the "Top 10 plant viruses" in a recent molecular pathology review (Scholthof *et al.,* 2011).

Seed transmission of CMV has been reported in many plant species, with efficiencies varying from less than 1% up to 50%. Horizontal transmission of CMV is vectored by aphids in a non-persistent manner and over than 80 species of aphids have been reported to transmit CMV (Palukatis *et al.,* 1992). CMV has icosahedral particles, approximately 28-30 nm in diameter. RNA1 and RNA2 are encapsidated in different particles, whereas RNA3 and RNA4 are probably packaged together in the same particle. Virus particles also contain low levels of the RNA species designated RNA4A, RNA5 and RNA6 (Garcia-Arenal & Palukatis, 2008). The genome of CMV consists of five genes distributed over three, single-stranded, positive-sense, capped, genomic RNAs (RNA1, RNA 2 and RNA3). RNA1 and 2 encode the nonstructural proteins involved in the viral replication: RNA1 (3.3–3.4 kb) is monocistronic and codes for c. 111 kDa 1a

protein which has a methyltransferase domain in its N-terminal part and a helicase motif in the C-terminal part. RNA2 (3.0 kb) is bicistronic and encodes for two protein: the 98 kDa 2a protein, which contains the conserved GDD motif of many RNA-dependent RNA polymerase, and the 13–15 kDa 2b protein, which is translated from a 630–702 nts subgenomic RNA designated RNA 4A that is co-terminal with the 3' end of RNA2. The ORF expressing the 2b protein overlaps with the ORF encoding the 2a protein, but it is in a +1 reading frame. The CMV 1a and 2a proteins have also function in promoting virus movement in several host species and 2b protein is involved in RNA interfering (RNAi) pathway and also influences virus movement in some hosts. RNA3, also bicistronic, encodes for 30 KDa 3a protein (viral movement protein, MP), essential for both cell-to-cell as well as long-distance (systemic) movement, as well as for the 25 kDa 3b protein (capsid protein, CP) which is expressed from a 1,010–1,250 nts subgenomic RNA, designated RNA4, that is co-terminal with the 3' end of RNA3. The 3b protein is also required for cell-to-cell mand long-distance movement, although the ability to form virions is not a requirement for movement (Palukatis *et al.,* 1992; Garcia-Arenal & Palukatis, 2008). The 224–338 nts 3' nontranslated regions of all three genomic and both subgenomic RNAs are highly conserved, forming a tRNA-like structure as well as several pseudoknots. The 5' nontranslated regions of RNA1 (95–98 nts) and RNA2 (78–97 nts) are more conserved in sequence with each other than with those of RNA3 (96–97 nts or 120–123 nts) (Boccard and Baulcombe, 1993). CMV also produces an RNA5 of unknown function(s), which is co-terminal with the 3' non-translated regions of RNA1 and RNA2 and is uncapped (Blanchard *et al.*, 1996; Melissane de Wispelaere, 2009). Its initiation nucleotide is in a 21 nts conserved sequence that is presnt in CMV members of subgroup II and absent in members of subgroup I (Thompson *et al*., 2008).

Symptom attenuation has been proposed based on the observation that the presence of more RNA5 in plants results in less severe symptoms (Shi *et al.,* 2007) and it has been suggested that RNA5 could be directly involved in virus assembly and/or replication (Blanchard *et al.,* 1996; Gould *et al.,* 1978). CMV particles also encapsidated a low level of tRNAs, which have been reported in the literature as CMV RNA6 (Garcia-Arenal & Palukatis, 2008).

Phylogenetic studies reveal that the CMV isolates can be divided into two main subgroups, I and II and moreover subgroup I can be further divided into IA and IB depending on the sequence of the 5' UTR of the genomic RNA 3 (Palukaitis *et al*., 1992; Wahyuni *et al.,* 1992). These differences are important to distinguish the virulence of the different strains of CMV and usually the subgroup I is more virulent than subgroup II. The two subgroups develop also several symptoms, the first show mosaic and stunt, whereas the second show very mild symptoms or asymptomatic infection. (Tomofumi Mochizuki & Satoshi T. Ohki 2012). RNA4A and RNA5 are only encapsidated by subgroup II strains and not in subgroup I (Thomson *et al.,2008*; Garcı´a-Arenal & Palukatis, 2008). CMV can also support satellite RNAs varying in size from 333 to 405 nts. These satellite RNAs are dependent upon CMV as the helper virus for both their replication and encapsidation, but have sequence similarity to the CMV RNAs limited to no more than 6–8 contiguous nucleotides. More than 100 satellite variants have been found associated with over 65 isolates of CMV from both of the subgroups. These satellite RNAs usually reduce the accumulation of the helper viruses and on most hosts reduce the virulence of CMV. However, this attenuation of disease is not due to competition between the helper virus and the satellite RNA for a limited amount of replicase or capsid protein. Certain satellite RNAs in some selected hosts can enhance the disease induced by CMV (Garcia-Arenal & Palukatis, 2008).

In this paper we describe the detection of CMV isolated from *A. chinensis* and *A. deliciosa* plants. Biological and molecular chararcterization of the kiwifruit isolate showed high amino acid identity with strains of CMV belonging to the subgroup I. Moreover, agroinfectious viral clone of the three RNAs of CMV have been produced in order to support further studies regarding the behavior of the virus in kiwifruit plants.

## Materials and methods

### Source plants

Plants of *A. chinensis* showing virus-associated symptoms were collected (sample K35) during the 2010 season from an orchard in Faenza, Emilia Romagna region. The plants expressed a yellow mosaic and pale mottle symptoms on the leaves. Moreover a female and male plants of *A. deliciosa* (K107 and K108) showing symptoms of leaf

mosaic were collected during the 2012 season from a nursery in Cesena, Emilia Romagna region.

**Transmissions to herbaceous indicators**

Sap extracts from symptomatic leaves of *A. chinensis* were mechanically inoculated on indicator plants such as *Nicotiana bentamiana*, *N. tabaccum* cv "Samsun"*, N. glutinosa, N. occidentalis*, *Phaseolus vulgaris, Chenopodium quinoa* and *C. amaranticolor*. Leaves tissue from actinidia were homogenized in a mortar adding 0.1 M Na-phosphate buffer pH 7.5, containing 0.12% sodium sulphite and 5% polyvinylpyrrolidone (Clover *et al*., 2003). The homogenate was mixed with celite powder and mechanically inoculated on leaves of herbaceous indicators that were then grown in a greenhouse at 20-22°C.

**Purification of viral particles and determination of viral protein weight**

The virus purification was performed following the protocol of Turina *et al*., (2007) with some modifications.

The frozen leaves of *N. bentamiana* (100 g wet weight) were homogenized in a blender in two volumes of K-phosphate buffer 0.25M pH 7.0 with the addition of 1% of sodium metabisulfite and 1mM of EDTA. After filtration through cheesecloth, the homogenate was added with 1% of Triton and stirred at 4°C for 1h. The homogenate was centrifuged at 9,300 x g for 20 min then the supernatant was subjected to ultracentrifugation in a Beckman 35Ti rotor at 95,000 x g for 5 h (6 tubes x 60ml). Each resulting pellet was resuspended overnight in 1 ml of K-phosphate buffer 0.25M pH 7.0. After centrifugation (9,300x g for 20 min), the supernatant was layered onto a 10 ml 20 % sucrose cushion prepared in the same buffer and centrifugated at 250,000 x g for 2h in a 60 Ti rotor (Beckman) (1 tube x 25 ml). The resulting pellet was dissolved in 500 µl of K-phosphate buffer 0.25M pH 7.0, loaded at the top of a 10%-50% sucrose gradient and centrifuged at 250,000 x g for 1 h 30 min in a SW41Ti rotor (Beckman).

A single band at 4.5 cm from the bottom of tube (Figure 3.2a) was collected, diluted in K-phosphate buffer 0.25M pH 7.0 and centrifuged at 250,000 x g for 2h. The resulting pellet was resuspended in 100 µl of K-phosphate buffer 0.25M pH 7.0 and purity of the viral suspension was checked by transmission electron microscopy. The suspension (20

µl) was placed on a carbon-coated electron microscopy grid for 10 min, wash with approximately 30 drops of water and stained with 1% uranyl acetate. Grids were examined using Philips CM10 apparatus.

Molecular weight of viral proteins was determined by separation on a 12% sodium dodecylsulfate-polyacrylamide gel (SDS-PAGE ) stained by Coomassie brilliant blue.

**Random RT-PCR and sequencing**

Viral nucleic acids were extracted from the purified virus particles with TRIzol® Reagent following the manufacturer's protocol. RT-PCR was carried out by a modification of Froussard protocol (Froussard, 1992). Briefly, 1 µl RNA was mixed with 1.5 µl of Universal primer-dN6 (10mM) (5'-GCCGGAGCTCTGCAGAATTCNNNNNN-3') in a total volume of 5 µl, heated at 70 °C for 5 min and rapidly cooled on ice. A mixture of 4 µl ImProm-II 5x-reaction buffer, 1.2 µl $MgCl_2$ (25 mM), 1 µl dNTPs (10 mM), 0.5 µl RNasin Ribonuclease Inhibitor (40 U/µl) (Promega, Madison, WI), 1 µl ImProm-II™ Reverse Transcriptase (Promega, Madison, WI) and 7.3 µl of nuclease-free water was incubated at 25°C for 5 min, 42°C for 1 h and 70°C for 15 min.

The reaction was boiled for 2 min and cooled in ice then, for the synthesis of the second-strand of cDNA the following reagents were added: 5 µl Klenow buffer 10x, 1.25 µl dCTP (100mM), 1.6 Klenow fragment (5 U/µl) (Promega, Madison, WI) and distilled water up to 50 µl. The mix was incubated 1 h at 37° C then purified with Wizard® SV Gel and PCR Clean-up System (Promega, Madison, WI) and eluted in 50 µl. Amplification of double-stranded cDNA (2 µl) was obtained in a volume of 25 µl containing 5 µl 5X Green GoTaq® Reaction Buffer, 1.5 µl of $MgCl_2$ (25 mM), 1.0 µl dNTPs (10 mM), 1.5 µl of Universal primer (10mM) (5'- GCCGGAGCTCTGCAGAATTC-3') and 0.25 µl GoTaq® (5 U/µl). The samples were then subjected to 94°C for 1 min and then to 40 cycles of 94°C for 10 sec, 55°C for 10 sec and 72°C for 3 min. PCR products were analyzed by 1% agarose gel electrophoresis and stained with ethidium bromide. For cloning, DNA was purified from excised bands using Wizard® SV Gel PCR Clean-Up System kit (Promega, Madison, WI) according to the manufacturer's protocol, ligated into pGEM T- easy vector (Promega, Madison, WI) and cloned in *E. coli* M1022 competent cells. Recombinant plasmids DNA were extracted with Wizard® Plus SV

Minipreps DNA Purification System, (Promega, Madison, WI) and sequenced by the company Eurofins MWG Operon (Germany).

**Circular RT-PCR**

To determine the sequence at 5' and 3' ends, a circularization RT-PCR procedure (Coutett *et al,* 1997) was applied as follows: 10 µg of RNA was incubated with 2.5 units of tobacco acid pyrophosphatase (TAP; Epicentre Technologies, Madison WI), 20 units of RNasin Ribonuclease Inhibitor (Promega, Madison, WI) and 2 µl of 10X TAP Reaction Buffer in a total volume of 20 µl. After incubation for 1 h at 37°C, RNA was precipitated with ethanol. In a total volume of 400 µl, 4 µg of decapped RNA was self-ligated with 20 units of T4 RNA ligase (Epicentre Technologies, Madison WI), 20 units of RNasin Ribonuclease Inhibitor (Promega, Madison, WI), 33 mM Tris acetate (pH 7.5), 66 mM potassium acetate, 10 mM magnesium acetate, 20 mM dithiothreitol, 100 µM ATP and 100 µg/ml acetylated BSA. After incubation for 16 hours at 16°C, self-ligated RNA was purified by phenol-chloroform, precipitated by ethanol and then resuspended in 10 µl of TE buffer. Synthesis of cDNA from each RNAs was obtained through reverse transcription using ImProm-II™ Reverse Transcriptase (Promega, Madison, WI) as described above and reverse specific primers binding the 5' end of each RNAs (Table 3.1). The PCR amplification was performed, using primer pairs specific for each RNA-1,-2 and -3 (Table 3.1) in a total volume of 25 µl containing 5 µl of cDNA, 3 units of *Pfu* DNA Polymerase (Promega, Madison, WI), 20mM Tris-HCl (pH 8.8), 10mM KCl, 10mM $(NH_4)_2SO_4$, 2mM $MgSO_4$, 0.1% Triton® X-100, 0.1mg/ml nuclease-free BSA, 400 µM of each dNTPs and 400 nM of each primer. Amplification steps were as follow: 94°C for 5 min followed by 35 cycles of 94°C for 1 min, 56°C for 1 min and 72°C for 1 h. The amplified DNA was analyzed in 1% agarose gel electrophoresis and stained with ethidium bromide. The amplicons obtained using each RNA specific primer pair, were purified from agarose gel, cloned in to pGEM-T Easy Vector and sequenced as described above.

**Construction of full-length agroinfectius clones and leaf agroinfiltration**

The full length amplification of RNA -1 -2 and -3 have been obtained using primer pairs specifically designed to bind 5' and 3' ends of the actinidia CMV isolate (Table 3.1) by ImProm-II™ Reverse Transcriptase and *Pfu* DNA Polymerase (Promega, Madison, WI) as described above with the exception, in the amplification phase, of the extension time that has been incremented to 4 minutes.

Amplicons obtained from each RNAs were ligated in the pJL89 binary vector previously digest with *Stu*I and *Sma*I enzymes. Ligate product was transformed by electroporation into *E. coli* M1022 competent cells then selected overnight on LB agar plates containing kanamycin (100µg/ml). Recombinant plasmids pJL1-8, pJL2-12 and pJL3-21, containing respectively the DNA sequence of CMV RNA-1, -2 and -3 from the actinidia isolate, were purified from LB broth culture and full-length sequence of each clone determined.

*Agrobacterium tumefaciens* cells (strain C58C1) were transformed by pJL1-8, pJL2-12 and pJL3-21 clones and selected on LB medium containing kanamycin (100µg/ml) and rifampicin (100µg/ml) and grown for 48 hours at 28 °C. The selected cells were grown overnight at 28 °C in 10 ml of LB medium containing the same antibiotics concentrations and plasmids collected by centrifugation. Each agroclones were resuspended in 5 ml of MA buffer (10 mM $MgCl_2$, 200 mM acetosyringone) adjusting the optical density at 600 nm (OD600 nm) to 0.6, mixed in equal amounts and left at room temperature for 3 h before leaf agroinfiltration in 3-week-old plants of *N. benthamiana* (Delbianco *et al*., 2013).

**Sequence alignment and phylogenetic analysis**

Based on the predicted amino acid sequence of the single proteins (1a, 2a, 2b, MP and CP), CLUSTAL W method in MEGA 6 software (Tamura *et al.* 2013) was employed to generate a multiple alignments and phylogenetic trees. Reference isolate sequences downloaded from GenBank database were included as representatives of CMV subgroups IA, IB and II. All phylogenetic analyses were carried out using the Minimum evolution method using Poison model with 1000 bootstrap replicates (Table 3.2).

## Results

### Symptoms observed and transmissions to herbaceous indicators

Systemic mosaic, necrotic local lesions and ringspot lesions were observed on leaves of *A. chinensis* female plant (sample K35) during the beginning of summer (July) 2010 (Figure 3.1a). Conspicuous yellow mosaic symptoms were also observed on leaves of *A. deliciosa* female and male plants (K107, K108) during the summer (July) 2012.

The sap extraction from symptomatic leaves of *A. chinensis* was inoculated to several herbaceous host and symptoms observed are listed in Table 3.2. The transmitted pathogen caused local chlorotic spots in *C. quinoa* and *C. amaraticolor* three days post-inoculation (dpi) that became necrotic 7 dpi. Systemic symptoms in the form of growth disorder, mosaic or mottling and deformation of young leaves were noticed on *N. glutinosa, N. tabacum* cv. "Samsun" *N. benthamiana* and *N. occidentalis* plants. Bright local symptoms were observed on *P. vulgaris* (Figure 3.1b)

**Table 3.2:** Reaction of test-plants, inoculated with *Cucumber mosaic virus* isolates from kiwifruit plant.

| Test Plants | Symptoms |
|---|---|
| *Chenopodium quinoa* | NLL |
| *Chenopodium amaranticolor* | NLL |
| *Nicotiana benthamiana* | SM |
| *Nicotiana glutinosa* | SM |
| *Nicotiana occidentalis* | SM |
| *Nicotiana tabacum* "Samsun" | SM |
| *Phaseolus vulgaris* | NLL |

*Note.* NLL: necrotic local lesion, SM: systemic mosaic

### Transmission electron microscopy (TEM) and determination of viral protein weight

A single band was collected from sucrose gradient (Figure 3.2a) and electron microscopy from virus purification showed isometric virus-like particles of about 28-30 nm (Figure 3.2b) corresponding to the typical particles of CMV isolates. Coomassie staining of SDS PAGE–separated purified virus evidenced the presence of one single polypeptide (Figure 3.3). The molecular weight, estimated on mobility relative to the molecular weight marker, was 26.5 kDa.


**Fig 3.1a:** Natural symptoms on *A. chinensins.* Chlorotic spot, mosaic and leaf yellowing were observed.

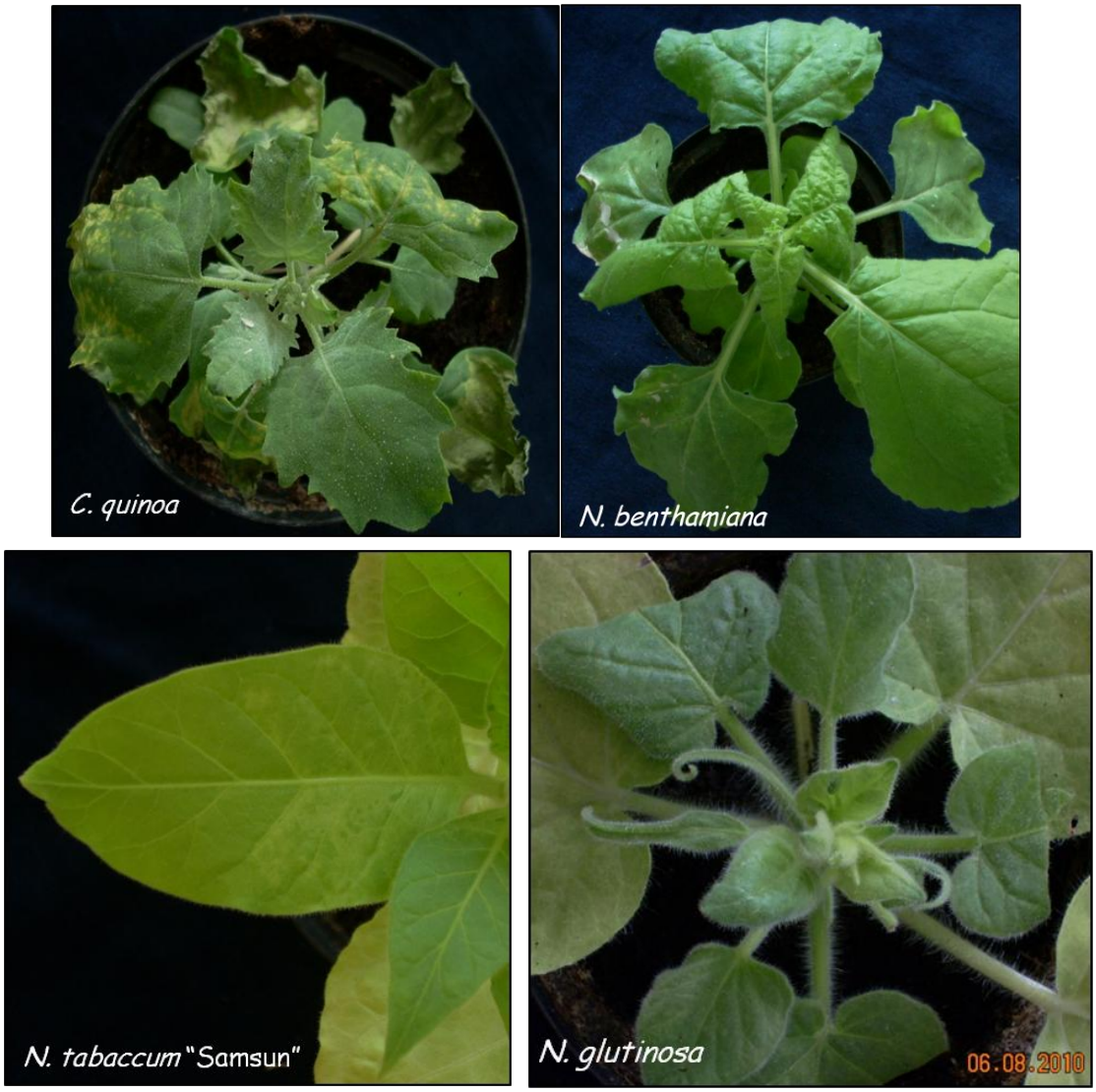



**Fig 3.1b:** Symptoms on indicator plants: local chlorotic spot on leaves of *C. quinoa* and systemic symptoms, mosaic or mottling and deformation of young leaves on *N. glutinosa, N. tabacum* cv. "Samsun" and *N. benthamiana.*

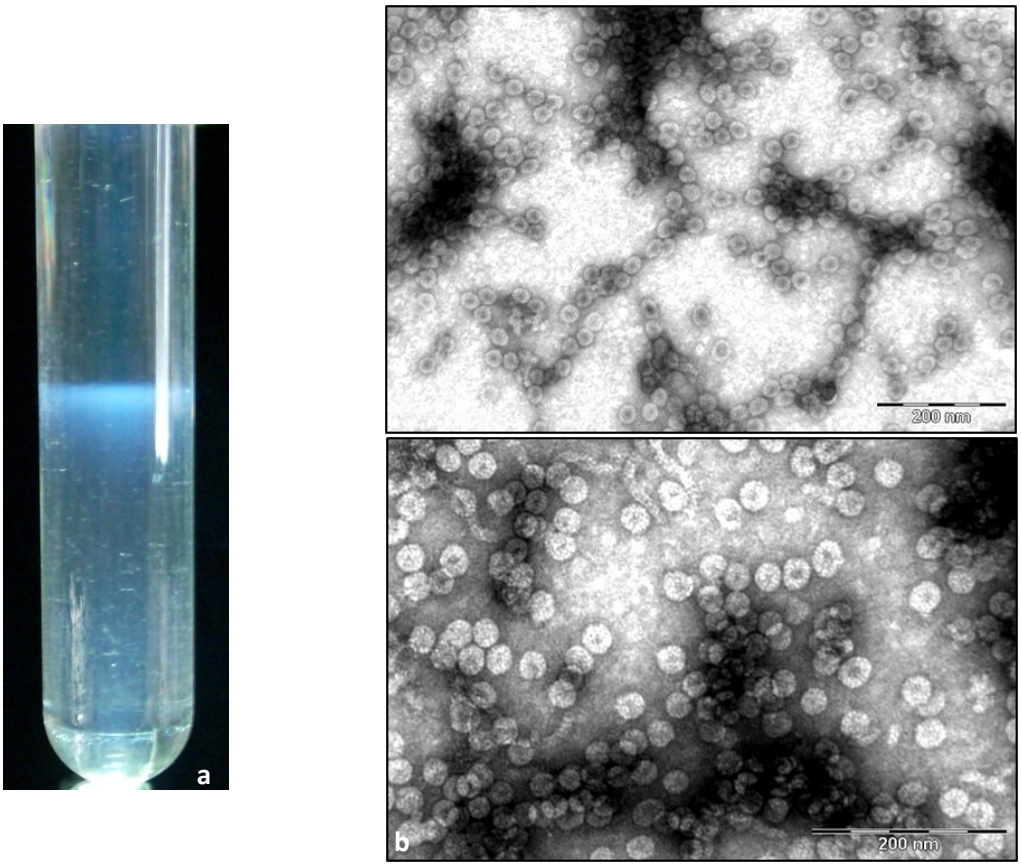



**Fig 3.2:** a) Band on sucrose gradient; b) negative staining of purified isometric virus-like particles of about 28-30 nm.

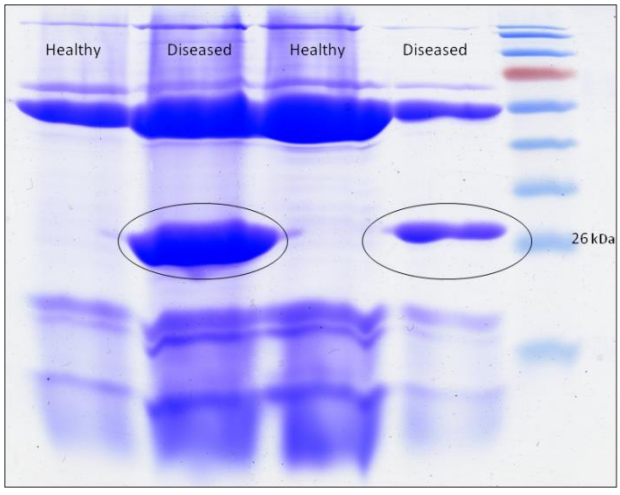



**Fig 3.3:** Coomassie staining of SDSPAGE–separated purified virus showing the presence of one single polypeptide.

**Sequencing**

Products analysis of random amplification done using the protocol from Froussard (1992) resulted in a pattern of multiple bands ranging from 200 to 1500 nucleotides in size. Sequences of selected cloned fragments evidenced high homology with CMV sequences of RNA1 and RNA2 regions encoding, respectively, for the protein 1a and 2b when blasted on GenBank database.

Three fragments, one from each of the genomic RNAs of CMV K35 isolate, were amplified by circular RT-PCR. Sequences analysis revealed that they resulted in the fusion of the last 204 nucleotides of 3' end followed by the first 154 nucleotides of 5' end for RNA1, the last 203 nucleotides of 3' end and the first 249 nucleotides of RNA2 5' end and the last 203 nucleotides of 3' end and the first 292 nucleotides at RNA3 5' end. On the basis of sequences obtained from 5' and 3' ends, specific primer pairs were designed and used to amplify and then clone full-length RNA1, 2 and 3.

RNAs of CMV K35 isolate resulted 3,358 (RNA1), 3,050 (RNA2) and 2,211 (RNA3) nucleotide long, showing the typical genome organization of the viral species.

RNA1 encoded for protein 1a (96-3077), RNA2 encoded for proteins 2a (518-3088) and 2b (2850-3179). RNA3 encoded for proteins 3a or movement protein (197-1036) and 3b or coat protein (1331-1987). Two additional fragments were amplified from circularized RNAs using primer pairs CMV1 3154-3173 F/CMV2 RNA4A R and CMV1 3154-3173 F/CMV3 RNA4 R (Table 3.1) that specifically targeted circularized CMV subgenomic RNA4a and RNA4, respectively. According to sequence obtained, nucleotide at positions 2,791 on RNA2 have been identified as the first nucleotides of subgenomic RNA4A which results 691 nucleotides long, while nucleotide at position 1,260 on RNA3 is the initiating point of subgenomic RNA4 (1024 nucleotides long).

cDNA obtained from circularized RNAs was used to investigate the presence of additional RNAs which are co-terminal with the 3' non-translated regions of RNA-1, -2 and -3. Amplification using primer CMV1 3154-3173 F (designed on conserved region between RNA1 to 3) in combination with primers pairs CMV1 3129-3150 R, CMV2 2823-2844 R or CMV R resulted, respectively in absence of amplification in the first one and in fragments of 302 and 286 nucleotides in the last two combinations. Sequences analysis indicated the nucleotides 2,745 on RNA2 and 1,990 on RNA3 as the starting point of additional RNAs.

**Table 3.1**: Primer uses on the characterization of CMV

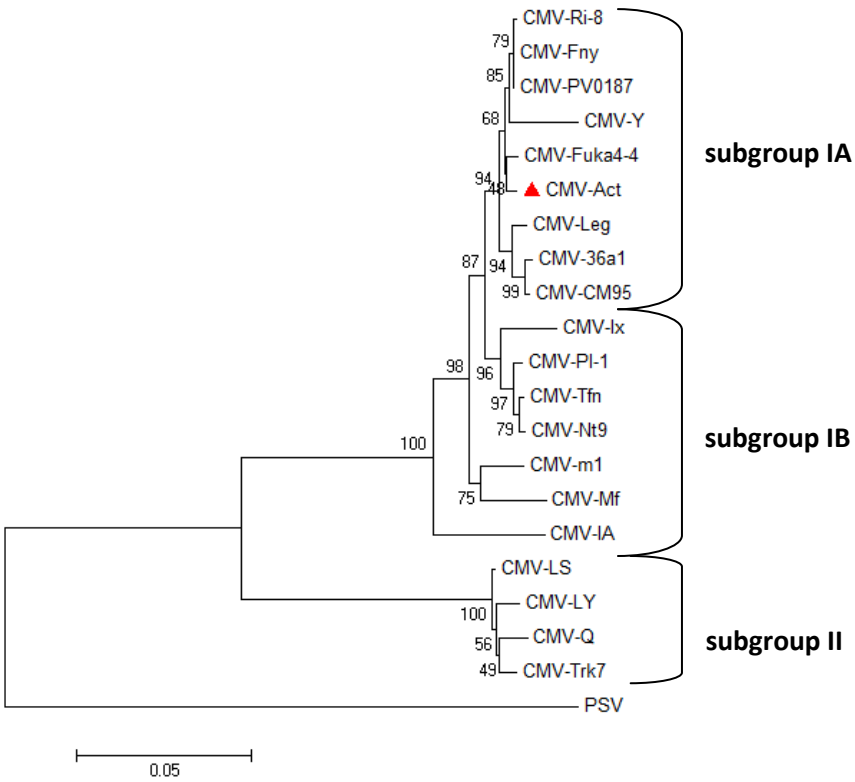
| Primer name | Sequence (5' – 3') | Position (nt) | Use |
|---|---|---|---|
| CMV1 3154-3173 F | GAACGGGTTGTCCATCCAGC | 3154-3173 | cRT-PCR |
| CMV1 135-154 R | TAGTCCTTTATCGCCGTGGG | 135-154 | |
| CMV2 230-249 R | TCTCGCTGACATCCACAGCG | 230-249 | |
| CMV3 273-292 R | AGGGGCCGGACTGAAATAGC | 273-292 | |
| CMV2 RNA4A R | ATTGCACCTACGTTCAATTCC | 2852-2872 | |
| CMV3 RNA4 R | ACTGGTTGATTCAGATTTGTCC | 1333-1354 | |
| CMV1 3129-3150 R | ACACAATGTGTTTAGTGACTTC | 3129-3150 | |
| CMV2 2823-2844 R | ACAATGTGTTTAGTGACTTCAG | 2823-2844 | |
| CMV1 1-23 F | GTTTTATTTACAAGAGCGTACGG | 1-23 | full-length |
| CMV1 end R | TGGTCTCCTTTTAGAGACCC | 3339-3358 | |
| CMV2 1-22 | GTTTATTTACAAGAGCGTACGG | 1-22 | |
| CMV2 end R | TGGTCTCCTTTTGGAGGC | 3464-3481 | |
| CMV3 1-23 F | GTAATCTTACCACTGTGTGTGTG | 1-23 | |
| CMV3 end R | TCCTTTTGGAGGCTCCCAC | 2265-2283 | |
| CMV F | GGA TGC TTC TCC ACG AG | 835 | Detection |
| CMV R | AGT GAC TTC AGG CAG T | | |
| sCMV-1 F* | GTAATACGACTCACTATAGGTTTT GTTTG | | |
| sCMV-2 R* | GGAATTCCCGGGTCCTG | | |

*Xi *et al*., 2006.

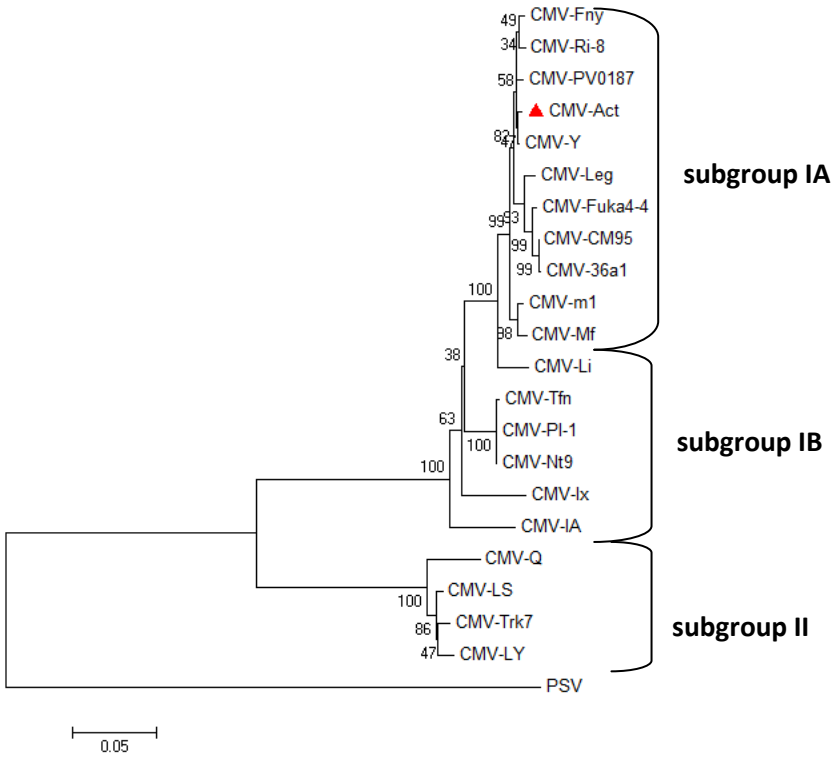
**Sequence alignment and phylogenetic analysis**

Sequences comparisons and phylogenetic trees were constructed for CMV isolated from actinidia (CMV-K35) based on the predict amino acid sequence of the 1a, 2a, 2b, MP and CP proteins. Divergence of members of subgroups I and II was observed in all phylogenetic trees and moreover, isolates of subgroup IA and IB were found on separate clades. The phylogenetic relationship between the amino acid sequences of

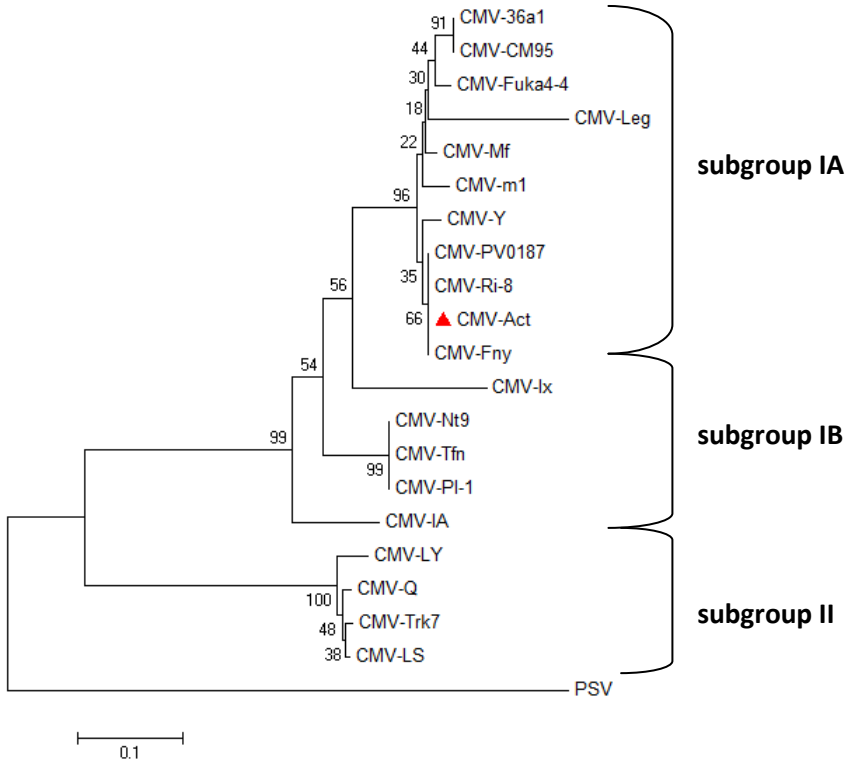
all the ORFs of CMV-K35 and those of other selected isolates available in GenBank, is shown in Figure 3.4a-e. The accession number and assigned abbreviations of these isolates are listed in Table 3.2. CMV-K35 clustered together with subgroup IA isolates. In particular protein 1a from CMV-K35 is closely related to the strain CMV-Fuka4-4 (isolated from cucumber in Japan). The protein 2a from CMV-K35 is closely related to strains CMV-PV0187 (from Germany) and CMY-Y (from Japan) while protein 2b from CMV-K35 is related to strains CMV-Ri-8 (isolated from tomato in Spain) and CMV-Fny (from United States). Movement protein and coat protein from CMV-K35 are closely related to strains CMV-PV0187 and CMV-Mf (From South Korea), respectively. Comparison of amino acid identity between sequence of all the ORFs from CMV-K35 and other strains revealed a homology of 88-91% with members of subgroup II, 92-95% with members of subgroup IB and 96-99% with members of subgroup IA. According to the phylogenetic analyses, the highest homologies (99%) of 1a, 2a, 2b, MP and CP ORFs of CMV-K35 were found with strains CMV-Fny, CMV-PV0187, CMY-Y, CMV-Fuka4-4, CMV-Mf, respectively.



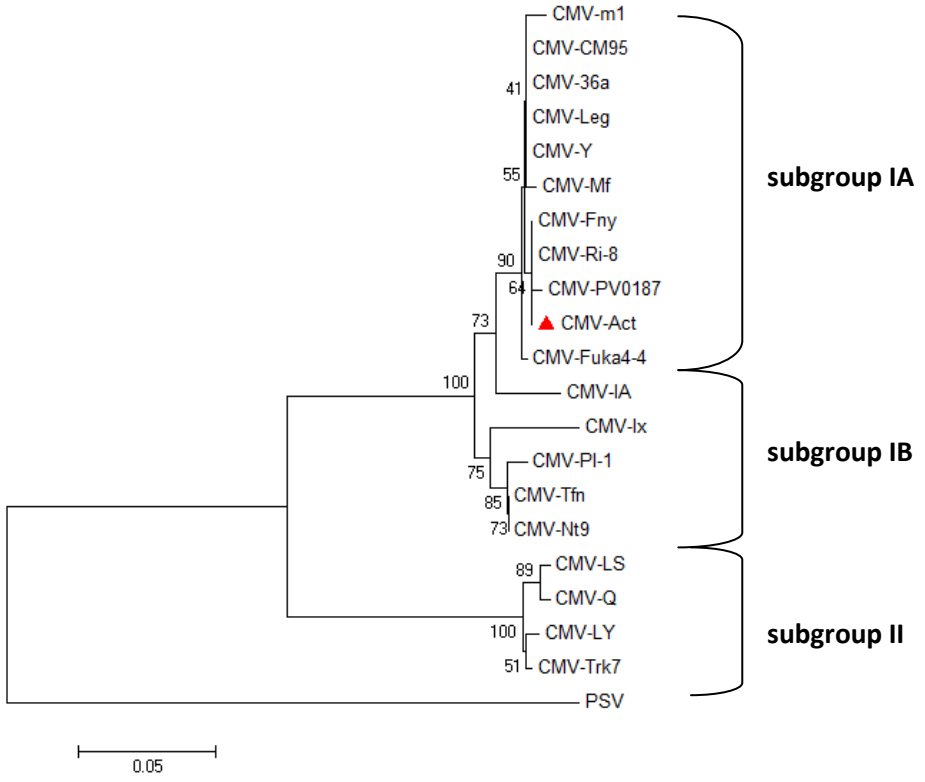

a ) Protein 1a



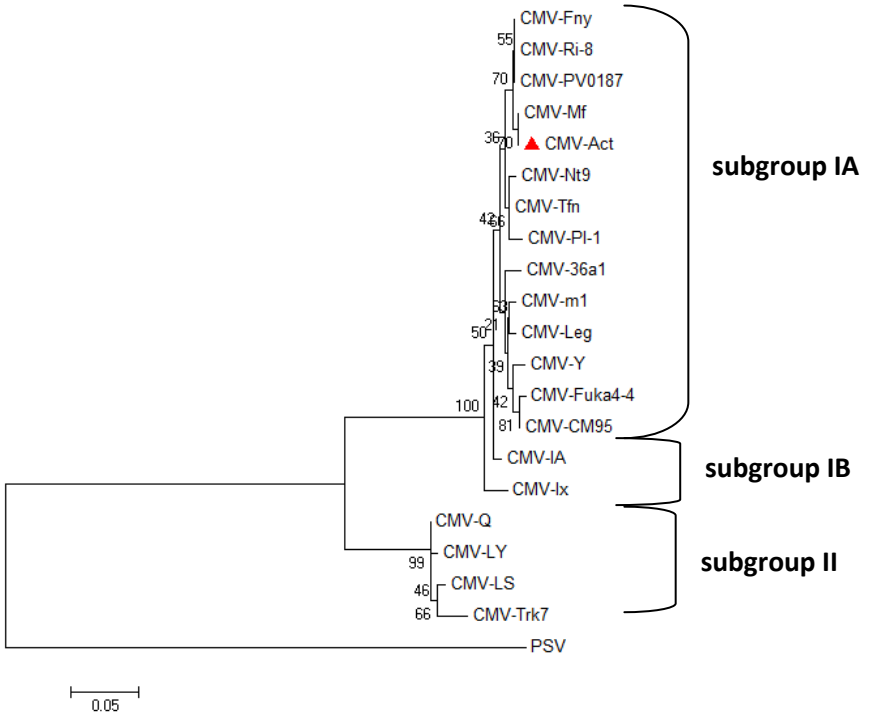

a) Protein 2a




b) Protein 2b




c) Protein MP




d) Protein 3b

**Fig. 3.4:** Phylogenetic trees of amino acid sequences inferred by Minimum Evolution method of the (a) protein 1a, (b) protein 2a, (c) protein 2b, (d) movement protein and (e) coat protein of CMV from actinidia and other isolates. Bootstrap values are shown as percentages. *Peanut stunt virus* (PSV) was used as the outgroup in trees (NC002038, NC002039, NC002040).

**Detection by RT-PCR**

By the use of primers CMVF/CMVR, that amplify a region of 835 nucleotides from RNA3 corresponding to the 3b protein (CP), the presence of CMV has been tested in tissues of *A. chinensis* and indicator plants. Positive results were also obtained testing *A. deliciosa* kiwifruit male and female plants (sample K107 and K108 respectively).
Moreover, all symptomatic indicator plants, including *N. benthamiana* plants infected by agroclones, tested positive to CMV.
Absence of satellite RNAs associated to CMV isolate K35 has been demonstrated by the use of the primers pair sCMV-1 F / sCMV-2 R (Xi et al., 2006) as all assayed samples resulted negative.

**Agroinfectious clones**

Leaf agroinfiltration of *N. benthamiana* by agrobacteria cells carrying a combination of pJL1-8, pJL2-12 and pJL3-21 clones, resulted in appearance of systemic symptoms 1 week after agroinfiltration showing typical distortion on non-infiltrated leaves, identical to those induced by wild type isolate K35. Together with positive results obtained by RT-PCR assay these data demonstrated that agroclones produce viable viral progeny able to infect experimental host as wild type isolate. Different attempts have been performed to use agroclones to infect *A. chinensis* and *A. deliciosa* plants through infiltration of leaf tissues or directly providing *A. tumefacines* cells carrying CMV clones, to wounds artificially produce on canes or trunk of 1 or 2 years old plants. No CMV infection has been detected as no symptoms have been observed and no RT-PCR positive results have been obtained from inoculated plants.

## Discussion

In this paper we reported the genome sequences of *Cucumber mosaic virus* infecting *A. chinensins.* The first indication that CMV can infect *Actinidia* sp has been reported in 1987 when Caciagli et al. (O, 1987) tried to test the reactions of *A. deliciosa* to some common viruses by mechanical inoculation. In their work only three viruses, *Tobacco necrosis virus* (TNV), *Tobacco rattle virus* (TRV) and the CMV induced symptoms on the inoculated leaves of the kiwifruit but only CMV gave systemic infection showing leaf chlorosis and reduced growth. After 27 years this is the first description of CMV that infected naturally *Actinidia* spp. in Italy. Systemic mosaic, necrotic local lesions and ringspot lesions were observed on leaves of *A. chinensis* (female plant). The hypothetical pathogen was successfully transmitted by mechanical inoculation to all the inoculated plants (*C. quinoa*, *C. amaraticolor, N. glutinosa, N. tabacum* cv. "Samsun", *N. benthamiana* and *N. occidentalis*) showing symptoms of necrotic local lesion, systemic mosaic and malformation of leaves (Figure 1b). By the molecular analyses this viral pathogen has been identified as CMV. Analysis of CMV-K35 sequences indicates that it is closely related to previously characterized isolates and that it is not a distinct variants specifically adapted to actinidia, as in the case of ASGV infecting kiwifruit that resulted a new strain of this species (Clover *et al.,* 2003). In particular, CMV-K35 showed highest levels of amino acid identity with CMV subgroup I isolates. Studies conducted worldwide have shown that the numerous CMV strains are classified into subgroup I and II and the subgroup I is further divided into IA and IB (Palukaitis *et al*., 1992; Wahyuni *et al.,* 1992). Strain IA and II of CMV are distributed worldwide, whereas the strains of IB subgroup are mainly restricted to Asia (Roossink, 2002; Koundal *et all.,* 2011). The correct subgroup identification is of fundamental importance, not only for epidemiological studies but also because it can be correlated with different biological properties of each isolate, such a symptomatology induced in a particular host and aphid transmissibility. Phylogenetic analysis of the single five ORFs (1a, 2a, 2b, MP and CP) showed that CMV-K35 was closely related to strains CMV-Fny, CMV-PV0187, CMY-Y, CMV-Fuka4-4 and CMV-Mf belong to the subgroup IA. Usually members of subgroup I induces more several symptoms in the field than subgroup II and thus it is easily recognized visually (Parrella & Sorrentino, 2009). This fact could explain the systemic mosaic, necrotic local lesions and ringspot lesions

observed on leaves of *A. chinensis.* Moreover, the same virus was detected also in plants of *A. deliciosa* from a nursery in Cesena (Emilia-Romagna region). The plants showed necrotic local lesions and ringspot. CMV has caused epidemics diseases in many crops in the world (Palukaitis *et al.,* 1992) and it is difficult to control due to its wide host ranges and rapid spread by its aphid vectors. In order to minimize the possibility of serious viral disease in kiwifruit it is therefore vital to use virus-free propagation material in order to prevent the spread of this virus. This virus is easily mechanically transmitted and while precautions should be taken not to spread it by this means, attempted elimination of this virus is unlikely to succeed because of the high probability of natural reinfection via aphids.

Up to date CMV RNA 5, a mixture of the 3' terminal regions of RNAs 2 and 3 (Blanchard et al., 1997; Shi et al., 1997), has been reported only in members of the subgroup II and its accumulation is correlated to the presence, within the members of the subgroup, of a 21 nucleotides conserved motif (named Box1), downstream the starting point of RNA5 (Palukatis *et al.,* 1992; Thompson et al., 2008). Exactly the same sequence has been also detected in some members of the *Benyvirus* genus, such as *Beet necrotic yellow vein virus* (BNYVV), where resulted involved in both long-distance movement and stabilization of the RNA3 decay product (Ratti *et al.*, 2009; Peltier *et al.*, 2012). Moreover, according to our results obtained by circular ligation of RNAs, additional RNAs co-terminal with the 3' untranslated regions of RNA-2 and -3 are also present on CMV-K35 isolate. Taking all together these information we can speculate on the origin of RNA5 as a cleavage product of RNA2 and 3 leading to a stable non-coding RNA (ncRNA) that may play an important role in the viral infection as suggested for ncRNA3 of BNYVV (Peltier *et al.*, 2012). Moreover, as Box1 sequence is not present within CMV-K35 RNAs, maybe a different motif is involved in RNA5 accumulation in CMV isolates not included in the subgroup II.

Additionally, some CMV strains encapsidate subviral RNAs known as satellite RNAs (satRNA), which differ from the CMV genome by being dispensable for CMV replication (Simon *et al.,* 2004). SatRNAs can have different effects on CMV replication, pathogenesis and symptom expression, depending on the host plant and the CMV strain (Feng *et al.,* 2012).

In the isolate of CMV-K35 no detectable satRNA have been observed. Most of satRNAs were detected in solanaceae plants such as tomato and tobacco (Kouadio *et al.,* 2013) so maybe the strain of CMV detected in actinidia is unable to support the replication of satRNAs. Actually, no satRNAs have been detected in any of the strains with high sequence identity with CMV-K35.

Finally we produced agroinfectious viral clones from K35 isolate with the purpose to infect kiwifruit plants on which perform studies regarding natural transmission of the virus and validate detection methodologies. Using CMV agroclones, we were able to perform successful infection of *N. benthamiana* that could produce severe symptoms including leaf curly but all attempts to infect kiwifruit plants were unsuccessful. Further experiments are therefore needed to understand if CMV infection of *Actinidia* spp. also require some additional elements not yet identified.

**Table 3.2:** Abbreviations and Accession numbers of reference isolates.

| Strain | Accession Numbers | | | Subgroup |
|---|---|---|---|---|
| | RNA1 | RNA2 | RNA3 | |
| CMV-36a1 | AB079889 | AB079890 | AB079891 | IA |
| CMV-CM95 | AB188234 | AB188235 | AB188236 | IA |
| CMV-Leg | D16403 | D16406 | D16405 | IA |
| CMV-Fuka4-4 | AB188231 | AB188232 | AB188233 | IA |
| CMV-Y | D12537 | D12538 | D12539 | IA |
| CMV-PV0187 | KP165580 | KP165581 | KP165582 | IA |
| CMV-Ri-8 | AM183117 | AM183118 | AM183119 | IA |
| CMV-Fny | D00356 | D00355 | D10538 | IA |
| CMV-m1 | AB920561 | AB920778 | AB920779 | IA |
| CMV-Mf | AJ276479 | AJ276480 | AJ276481 | IA |
| CMV-Nt9 | D28778 | D28779 | D28780 | IB |
| CMV-PI-1 | AM183114 | AM183115 | AM183116 | IB |
| CMV-Tfn | Y16924 | Y16925 | Y16926 | IB |
| CMV-Ix | U20220 | U20218 | U20219 | IB |
| CMV-IA | AB042292 | AB042293 | AB042294 | IB |
| CMV-LY | AF198101 | AF198102 | AF198103 | II |
| CMV-Trk7 | AJ007933 | AJ007934 | AJ007935 | II |
| CMV-Q | X02733 | X00985 | M21464 | II |
| CMV-LS | AF416899 | AF416900 | AF416976 | II |

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# Chapter 4:
# Project Paper:

## First report of *Pelargonium zonate spot virus* infecting *Actinidia*

## First report of *Pelargonium zonate spot virus* infecting *Actinidia chinensis*

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## Abstract

*Pelargonium zonate spot virus* (PZSV) is the single member of the *Anulavirus* genus (Fam. *Bromoviridae*) first isolated from tomato in Italy and later reported also from Spain, France, USA and Israel. Up to now PZSV has known to naturally infect only herbaceous hosts as tomato, pepper, artichoke and common weeds often symptomless. Symptoms on leaves and fruits of infected tomato plants are characterized by line patterns, chlorotic and necrotic rings, together with plant stunting, leaf malformation and poor fruit set, which often result in plant death. During the season 2011 plants of kiwifruit *Actinidia chinensis* cv. Hort16A, exhibiting viral symptoms, were observed in two orchards in Faenza province, Emilia-Romagna region. Symptoms include chlorotic and necrotic rings on leaves and depressed areas on the fruits with consequently deformation of the berries. The causal agent has been successfully transmitted to indicator plants and RT-PCR analyses, performed using PZSV primer pair, specifically identified the virus in all indicator hosts and in leaves and fruits collected from all symptomatic kiwifruit plants. The new PZSV isolate (PZSV-Act) has been characterized by sequencing and by transmission electron microscopy investigations.

## Introduction

Kiwifruit (genus *Actinidia*) is an important horticultural crop grown in the temperate regions. The four world's largest producers are China (480,000 tons) Italy (450,049 tons), New Zealand (372,833tons) and Chile (230,333tons) (Belrose Inc., 2012). Currently more than 50 species and about 76 taxa are recognized in the genus, but the principal species in cultivation are *Actinidia delicosa, Actinidia chinensis* and *Actinidia arguta* (Li & Lowe, 2007).

Kiwifruit plant are considered to be relatively disease free, just some fungal infections were divulged in the past, like *Armillaria novae-zelandii* in New Zealand (Horner, 1992), *Cadofhora melinii* in Italy (Prodi *et al*., 2008), *Phomopsi* sp. in Greece (Elena, 2009) and verticillum wilt of gold kiwifruit in Chile (Auger *et al*., 2009). To date, these pathogens tend to be localized.

In the past few years severe damages in *A. chinensis* cv Hort 16A orchards were caused from a virulent strain of *Pseudomonas syringae* pv. *actinidiae* with adverse consequences for the production in Italy and New Zealand (Ferrante and Scortichini, 2010; Everett *et al.*, 2011). However, there are no documented cases of viral diseases of kiwifruit before 2003. After the increasing of the kiwifruit as commercial crop in Italy, Caciagli and Lovisolo (1987) surveyed commercial orchards for potential viral diseases and collected samples from 100 symptomless *A. deliciosa* and one plant of *A. deliciosa* that showed chlorotic mottling. The extracts from these plant were mechanically inoculated into four herbaceous indicators (*Chenopodium quinoa, Chenopodium amaranticolor, Nicotiana glutinosa* and *Nicotiana clevelandii*). None of the 404 inoculated indicator plant displayed symptoms. Moreover, the authors tested the susceptibility of *A. deliciosa* to some common viruses from Italy and only the CMV moved systemically and induced symptoms on the inoculated leaves of the kiwifruit.

In 2003, was reported the first definitive identification of a virus infecting *Actinidia* spp. as a strain of *Apple stem grooving virus* (ASGV) were detected in *A. chinensis* imported from China and held in New Zealand quarantine (Clover *et al.*, 2003).

Subsequent examination of kiwifruit germplasm from the same quarantine containment has detected several additional viruses, including a ~ 300 nm rigid rod related to *Ribgrass mosaic virus,* a Tobamovirus, (Chavan *et al.,* 2009; Pearson *et al.,*

2010; Pearson *et al.,* 2011;) and two novel vitiviruses *Actinidia virus A* (AcVA) and *Actinidia virus B* (AcVB) (Blouin *et al*., 2012).

Although to date no obvious viral problem have been reported in commercial crop but further examination of kiwifruit plant has detected additional viruses.

From *A. chinensis* was identified a novel putative *Potexvirus, Actinidia virus X* (Pearson *et al.,* 2011). Two viruses belong to the family *Bromoviridae, Alfalfa mosaic virus* (AMV) and *Cucumber mosaic virus* (CMV) were detected by ELISA, both have very wide host range and both were detected in *A. glaucophylla* and *A. fortunatii* and only AMV in *A. guilinensis* (Pearson *et al.,* 2011) while CMV was detected in *A. chinensis* and *A. deliciosa* in Italy (Biccheri *et al.,* 2015). In addition, Actinidia citrivirus, a proposed member of the genus *Citrivirus* (family *Betaflexiviridae*) was detected from *A. chinensis* (Pearson *et al.,* 2011; Chavan *et al.,* 2013) and another member of the genus *Tobamovirus* was detected in *A. chinensis,* the *Turnip vein clearing virus* (TCTV) (Chavan *et al.,* 2009). Finally, *Cherry leaf roll virus* (CLRV), family *Secoviridae,* has been detected in New Zealand on *A. chinensis* cv. Hort16A and also in *Rumex* spp. growing below the infected vines. CLRV on the other hand has been found to cause severe symptoms including leaf spotting, leaf necrosis, bark splitting, cane dieback and changes in fruit shape of actinidia plants (Woo *et al.,* 2012a ; Woo *et al.,* 2012b). Recently two new detection were described in China where *Actinidia virus A* and *Actinidia virus B* were detected on *A. chinensis* (Zheng *et al.,* 2014) and in India with the characterization of *Apple stem grooving virus* infecting *A. deliciosa* (Bhardwaj *et al.,* 2014).

In this paper we describe the characterization of a new virus infecting *A. chinensis, Pelargonium zonate spot virus* (PZSV) that is the type species of the *Anulavirus* genus within the *Bromoviridae* family (Bujarski *et al.,* 2012). PZSV has been reported on tomato, pepper and weed species from Italy, Spain, France, USA and Israel (Gallitelli, 1982; Luis-Arteaga and Cambra, 2000; Gebre-Selassie *et al*., 2002; Liu and Sears, 2007; Escriu *et al*., 2009; Lapidot *et al*., 2010).

As well as tomato, pepper and geranium, PZSV also infects, in nature, globe artichoke, *Capsella bursa-pastoris*, *Chrysanthemum segetum*, *Diplotaxis erucoides*, *Picris echioides* and *Sonchus oleraceus* and it has been transmitted to herbaceous plant in 29

species, within nine dicotyledonous families, by mechanical inoculation (Martelli & Cirulli, 1969; Gallitelli, 1982).

Viral particles of PSZV are non-enveloped and quasi-spherical, with a diameter from 25 to 35 nm and coat protein of about 23 kDa (Gallitelli *et al.,* 2005). Sequence of complete genome has been obtained from the Italian tomato isolate that is divided in three RNAs species encoding four proteins (Figure 4.1) (Finetti-Sialer & Gallitelli, 2003). RNA-1 is 3383 nts long, with a single ORF 1a encoding a polypeptide which contains conserved motifs of type I methyltransferases and of the helicases of superfamily 1. RNA-2 is 2435 nts long and encodes for a polypeptide (ORF 2a) showing identity to the RNA-dependent RNA polymerases of positive-strand RNA viruses. RNA-3 is 2659 nts long and contains two ORFs. The product of the first ORF 3a revealed similarities with the 30K superfamily of virus movement proteins. The second ORF 3b encodes the viral coat protein, which is expressed via the subgenomic RNA-4 (Finetti-Sialer & Gallitelli, 2003; Gallitelli *et al*., 2005).

Poor data are available on variability within PZSV isolates. High amino acid identity has been reported between Italian and Israeli tomato isolates (93% ORF 1a, 97% ORF 2a, 98% ORF 3a and 96 % ORF 3b) (Lapidot *et al*., 2010).

PZSV induces conspicuous concentric chrome-yellow bands in the leaves in *P. zonale* infected plant, from which derived its name and is the causal agent of a severe tomato disease characterized by concentric chlorotic/necrotic rings and line patterns of leaf stems and fruits together with plant stunting, leaf malformation and reduced fruit set, which often result in plant death (Gallitelli, 1982).

PZSV is seed-borne in *D. erucoides* and *N. glutinosa*. The virus is associated with the pollen carried by thrips that transmit it feeding on flowers of susceptible hosts (Vovlas *et al.,* 1989; Gallitelli *et al.,* 2005). In tomato PZSV is transmitted by seed, with efficiency of 29%, through pollen but infected pollen cannot horizontally transmit the virus to mother plant (Lapidot *et al*., 2010).

Recently, in contrast with previous reports exclusively from herbaceous hosts, we have detected and characterized PZSV in several symptomatic kiwifruit plant, in Italy.

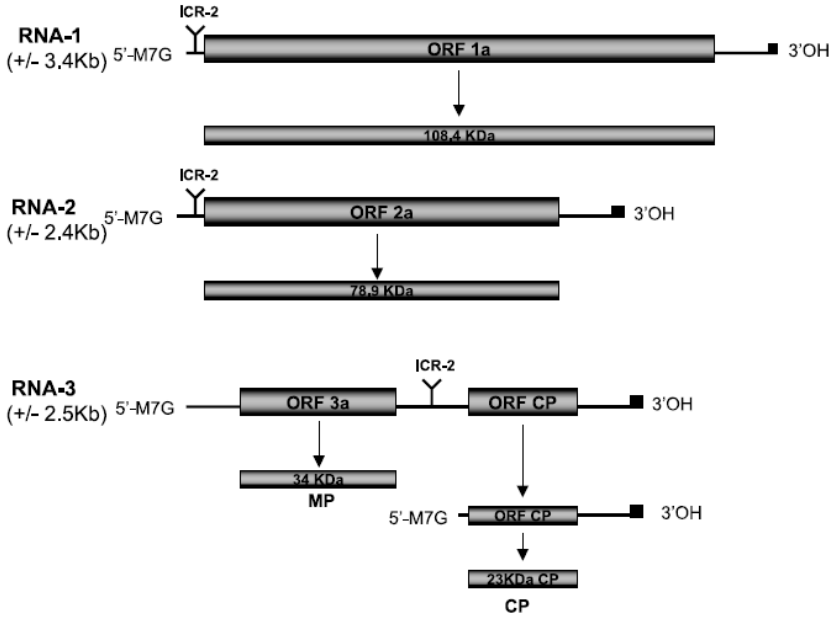



**Fig 4.1:** Genome structure of RNA1, RNA2 and RNA3 of *Pelargonium zonate spot virus.* Source: Galitelli *et al.,*2005.

## Materials and methods

### Virus isolates and host plant observation

Plants of *A. chinensis* cv. Hort16A exhibiting viral symptoms (sample K75), were observed in two orchards in Faenza province, Emilia-Romagna region, in May 2011. Symptoms include chlorotic and necrotic rings on leaves and depressed areas on the fruits with consequently deformation of the berries. Four infected plants have been identified during 2011 and 3 additional plants in 2012. In addition cuttings were obtained from symptomatic plants. Portions of canes 25-30 cm long were dipped in the acid 3-indolebutyric (200 ppm) for 5 min and then sticked in small pots contained sterilized soil for at least 60 days. Cuttings were grown in a greenhouse at 20-22°C with high levels of umidity.

### Transmissions to herbaceous indicators

Sap extracts from leaves of *A. chinensis* with presumed viral symptoms were mechanically inoculated on indicator plant as *N. bentamiana*, *N. tabaccum, N. glutinosa and C. quinoa*. Leaves tissue from *A. chinensis* were homogenized in 0.1 M Na-phosphate buffer, pH 7.5 containing 0.12% sodium sulphite and 5%

polyvinylpyrrolidone in a mortar (Clover *et al*., 2003). The homogenate was mixed with celite powder and mechanically inoculated on herbaceous indicators that were grown in a greenhouse at 20-22°C.

**Transmission electron microscopy (TEM)**

Leaf samples with viral symptom of approximately 0.25 g were collected from *C. quinoa* showing chlorotic local lesion. The sample was ground in 1 ml of K-phosphate buffer 0.1 M (pH 6·8) and 20 µl of the homogenate was placed on a carbon-coated electron microscopy grid for 2 min, washed with approximately 30 drops of water and stained with 1% uranyl acetate. Grids were examined using Philips CM10 apparatus transmission electron microscope

**Purification of the viral particles**

The virus purification was performed following the protocol of Turina *et al*. (2007) with some modifications.

The frozen leaves of *C. quinoa* (100 g wet weight) were homogenized in a blender in two volumes of K-Phospate buffer 0.25M with the addition of 1% of sodium metabisulfite and 1mM of EDTA. After filtration through cheesecloth, the homogenate was added with 1% of Triton and stirred at 4°C for 1h. The homogenate was centrifuged at 9,300 x g for 20 min, the supernatant was subjected to ultracentrifugation in a Beckman 35Ti rotor at 95,000 x g for 5 h (6 tubes x 60ml). Each resulting pellet was resuspended overnight in 1 ml of K-Phospate buffer 0.25M pH 7.0. After centrifugation (9,300 x g for 20 min), the supernatant was layered onto a 10 ml of 20 % sucrose cushion prepared in the same buffer and centrifugated at 250,000 x g for 2h in a 60Ti rotor (Beckman) (1 tube x 25 ml). The resulting pellet was dissolved in 500 µl of K-Phospate buffer 0.25M pH 7.0, loaded at the top of a 10%-50% sucrose gradient and centrifuged at 250,000 x g for 1 h and 30 min in a SW41Ti rotor (Beckman). Collected band was diluted in K-phosphate buffer 0.25M pH 7.0 and centrifuged at 60,000 x g for 2h. The resulting pellet was resuspended in 100 µl of K-phosphate buffer 0.25M pH 7.0 and purity of the viral suspension was checked by transmission electron microscopy. The suspension (20 µl) was placed on a carbon-coated electron

microscopy grid for 10 min, washed with approximately 30 drops of water and stained with 1% uranyl acetate. Grids were examined using Philips CM10 apparatus.

**Random-PCR amplification and sequencing**

RNAs from purified virus was extracted using TRI Reagent® (Sigma-Aldrich) following the manufacture's protocols. For random-PCR Froussard's protocol was followed (Froussard, 1992) with some modification. Briefly, for the synthesis of first strand, 1 µl total RNAs was mixed in 2.5 µl of distilled water and 1.5 µl of Universal primer-dN6 (10 mM) (5'- GCCGGAGCTCTGCAGAATTC$N_6$-3'), heated to 70 °C for 5 min and rapidly cooled on ice. To the previous mix 4 µl of ImProm-II 5x buffer (Promega, Madison, WI), 1.2 µl of $MgCl_2$ (25mM), 1.0 µl dNTPs (10 mM), 1.0 µl DTT (100 mM), 0.5 µl RNasin® (40u/µl) (Promega, Madison, WI), 1.0 µl ImProm-II™ Reverse Transcriptase (Promega, Madison, WI) were added. Incubation was at 25°C for 5min then 42°C for 1 h and 70°C for 15 min.

The reaction was boiled for 2 min and cooled in ice. The second-strand cDNA was obtained adding 5 µl Klenow buffer 10x (Promega, Madison, WI), 1.25 µl dCTP (100mM), 1.6 µl Klenow fragment (8 units) (Promega, Madison, WI) and 22.15 µl distilled water. The mix was left 1 h at 37° C and then the samples was purified with Wizard® Plus DNA purification system (Promega, Madison, WI). Two µl of DNA was amplified with GoTaq®DNA polymerase (Promega, Madison, WI), as follows: 5 µl Green GoTaq® Reaction Buffer 5X, 1.5 µl of $MgCl_2$ (25 mM), 1.0 µl dNTPs (mix 10 mM), 1.5 µl of Universal primer (10 mM) (5'-GCCGGAGCTCTGCA-3'), 0,25 µl GoTaq® DNA Polymerase (5u/µl) and distilled water until 25 µl.

The program consisted of 94°C for 1 min and then 40 cycles of 94°C for 10 sec, 55°C for 10 sec and 72°C for 3 min. PCR product were analyzed by 1% agarose gel electrophoresis and stained with ethidium bromide. For cloning, DNA was purified from excised bands using Wizard® SV Gel PCR Clean-Up System kit (Promega, Madison, WI) according to the manufacturer's protocol. The amplified products were ligated into pGEM T- easy vector (Promega, Madison, WI) and cloned in *Escherichia coli* M1022 competent cells. Recombinant plasmids DNA were extracted with Wizard® Plus DNA purification system (Promega, Madison, WI) and sequenced by external company (MWG-Biotech AG, Germany).

**Circular RT-PCR and full-length amplifications**

In order to determine the sequence of 5' and 3' ends we performed a RNA circularization and RT-PCR following the Coutett's procedure with some modifications (Coutett *et al.,* 1997). Ten µg of total RNAs were deccaped using 2.5 units of tobacco acids pyrophosphatase (TAP; Epicentre Technologies, Madison WI) and 20 units of RNasin® (40u/µl) (Promega, Madison, WI) in 20 µl of 50 mM sodium acetate pH 6.0, 1mM EDTA, 1% 2-mercaptoethanol and 0.1% Triton X-100 then incubated at 37°C for 1hr. After purification with phenol-chloroform, the RNA was precipitated with ethanol and resuspended in 10 µl of nuclease free water.

RNA ligase was performed as follows: in a total volume of 400 µl, 4 µg of deccaped RNA was incubated whit 20 units of T4 RNA ligase (Epicentre Technologies, Madison WI), 20 units of RNasin® (40u/µl) (Promega, Madison, WI), 50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 20mM dithiothreitol, 100 µM ATP and 100 µg/ml acetylated BSA and incubated for 16 h at 16°C. After phenol-chloroform extraction and ethanol precipitation the RNA was resuspended in 10 µl of nuclease free water.

Reverse transcription and PCR amplifications were performed using primers designed on published sequences of RNA 1-2 and 3 (Finetti-Sialer & Galitelli, 2003).

The RT was performed using 2 µl ligated RNAs and reverse primers PSZV 1 330-349 R, PZSV2 213-233 R and PZSV3 374-395R (Table 4.1) specific respectively for the RNA 1 - 2 and 3, following the procedure described above. The PCR amplification was made using reverse primers in combination with the forward primer PZSV 123F (Table 4.1) designed on a sequence shared within 3' UTR of all PZSV RNAs. The amplifications were carried out using the GoTaq® Long PCR Master Mix (Promega, Madison, WI) following the manufacture's protocol. The PCR's program consisted of 94°C for 5 min followed by 35 cycles of 94°C for 15 sec, 55°C for 15 sec and 72°C for 1.5 min. The amplified DNAs were analyzed in 1% agarose gel electrophoresis and stained with ethidium bromide. The amplicons around 400 bp of each cDNA were excised from the gel, purified with Wizard® SV Gel PCR Clean-Up System kit (Promega, Madison, WI) according to the manufacturer's protocol, ligated into the pGEM-T Easy vector (Promega, Madison, WI) which was subsequently used to transform competent cells of *E. coli* M1022 strain. Recombinant plasmids DNA from transformed cells was purified by Wizard® Plus SV Minipreps (Promega, Madison, WI) and sequenced.

Full-length amplification of each PZSV RNAs was performed using specific pairs of primers : PZSV RNA1endF - PZSV RNA1endR for full-length amplification of RNA 1; PZSV RNA2endF - PZSV RNA2endR for RNA 2 and PZSV RNA3endF - PZSV RNA3endR for RNA3 (Table 4.1).

**Table 4.1**: Primer used for PZSV characterization

| **Primer name** | **Sequence (5' – 3')** | **Position (nt)** |
|---|---|---|
| PZSV123F | TGAAGTAATTGAATGTGTTGGG | 3,320-3,341(RNA1)<br>2,433-2,454 (RNA2)<br>2,596-2,617 (RNA3) |
| PZSV1 330-349 R | TTTCGCACTGTCTCATAGCC | 330-349 |
| PZSV2 213-233 R | AACTGCATAAGTCCACTGTCC | 213-233 |
| PZSV3 374-395 R | TTTGAAGACTATTGTCCAGAGC | 374-395 |
| PZSV RNA3end F | GTTTGAACTTAGTAATTGCATGTG | 1-24 |
| PZSV RNA3end R | GTCTCTCTTAGAGAAACCGAAG | 2632-2653 |
| PZSV RNA2end F | GTTTGAGTGCATTTTGTGTATTTG | 1-24 |
| PZSV RNA2end R | GGTCTCTCTTAGAGAAACCGAAG | 2468-2490 |
| PZSV RNA1end F | GTTTGAGTGCATTTTGTGTATTTG | 1-24 |
| PZSV RNA1end R | GGTCTCTCTTAGAGAAACCG | 3356-3375 |
| PZSV3 MPF | ATGTCTCTGATTCGGCGCTCC | 335-355 |
| PZSV3 MPR | TCAAAAGAAGGCAGACTGCGTCG | 1242-1264 |
| PZSV3 CPF | ATGCCCCCTAAGAGACAGAACACTG | 1620-1644 |
| PZSV3 CPR | CTACAGAGGTATATACTCTGCTTGG | 2222-2246 |
| PZSV2 F | GATAAATTCAGAGCTCTCGG | 138-157 |
| PZSV2 R | ATCTCTGCAGATTGTGTTCC | 1115-1134 |

**Serological and molecular detection**

Double-antibody sandwich (DAS)-ELISA was carried out on leaves of *A. chinensis* plants showing symptoms and on the mechanically inoculated indicator plants (one weeks after inoculation) using antiserum raised against PZSV (ADGEN Phytodiagnostics, Scotland), according to the manufacturer's instructions. Dried leaves of *C. quinoa* infected with PZSV isolated from tomato were used as a positive control. Moreover the Dot blot DNA hybridization kit (Agritest, Valenzano, Italy) was used according to the manufacturer's instructions and previous report (Finetti-Sialer & Galitelli, 2003).

Primer pair PZSV2 F - PZSV2 R was employed for molecular detection by RT-PCR using ImProm-II™ Reverse Transcriptase and GoTaq®DNA polymerase (Promega, Madison, WI) as described above. The program consisted of 94°C for 1 min and then 30 cycles of 94°C for 10 sec, 55°C for 10 sec and 72°C for 1 min.

**Identification of the PZSV encoded suppressor of RNA silencing**

Individual ORFs coat protein (CP) and movement protein (MP), were amplified using specific primer pairs PZSVCP F - PZSVCP R and PZSVMP F - PZSVMP R (Table 4.1) for CP and MP, respectively, by *Pfu* polymerase (Promega, Madison, WI) according to the manufacturer's protocols. The amplified fragments were cloned into the binary expression vector pBIN61 under the control of the *Cauliflower mosaic virus* (CaMV) 35S promoter and transformed in *Agrobacterium tumefaciens* strain C58C1 to obtain pBIN61-CP and pBIN61-MP clones. An overnight culture of each clone was mixed in equal volumes (final OD600= 0.6) with a culture of the clone pBIN61-GFP expressing the Green Fluorescent Protein (GFP) (Himber *et al*., 2003). The mixture was co-infiltrated into transgenic *N. benthamiana* plants (line 16C) carrying a highly expressed GFP transgene (Voinnet & Baulcombe, 1997; Voinnet *et al.,* 1998). GFP expression was observed under UV light.

## Results

### Symptoms on host plant

Plants of *A. chinensis* cv. Hort16A showing chlorotic and necrotic rings on leaves and depressed areas on the fruits with consequently deformation of the berries were collected in Emilia Romagna region (Figure 4.2). Symptoms appear early in the spring and remain evident until the end of the season in plant with severe infection but disappear at the beginning of summer in plant with mild or sectorial infection. The symptomatic plant, held under observation, showed year by year, a decreasing of vigour and then of productivity until the complete death of the scion (*A. chinensis* cv. Hort16A) but not of the rootstock (*A. deliciosa* cv. Hayward). Moreover cuttings was obtained from symptomatic plants but to date are still symptomless.

### Transmissions to herbaceous indicators

Symptoms were observed on mechanically inoculated plants of *N. bentamiana*, *N. tabacum, N. glutinosa and C. quinoa*. In particular *C. quinoa* plants showed local chlorotic lesions 3 day post-inoculation and one week post-inoculation were observed chlorotic or necrotic local lesions, systemic mosaic and marginal necrosis in *N. glutinosa* plants, chlorotic rings, systemic mottling on *N. tabacum* and *N. bentamiana* plants. The virus was maintained on *C. quinoa* (Figure 4.3a).

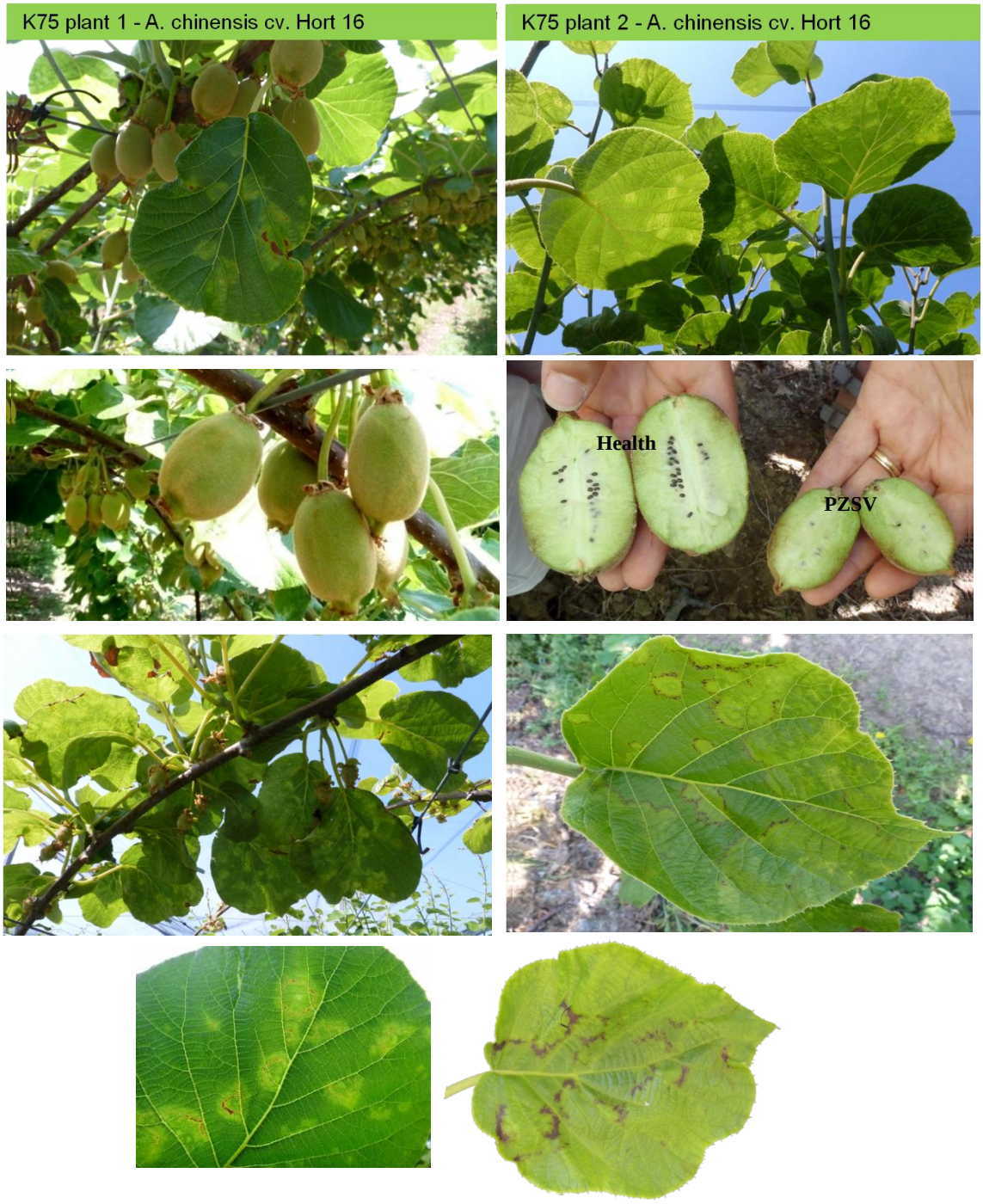



**Fig 4.2:** Plants of *A. chinensis* cv. Hort16A exhibiting chlorotic and necrotic rings on leaves and depressed areas on the fruits with consequently deformation of the berries.

**Purification of the Viruses**

Viral particles with diameter of about 30 nm and with quasi-spherical morphology were observed on viral purification obtained from *C. quinoa* infected leaves (Figure 4.3b)

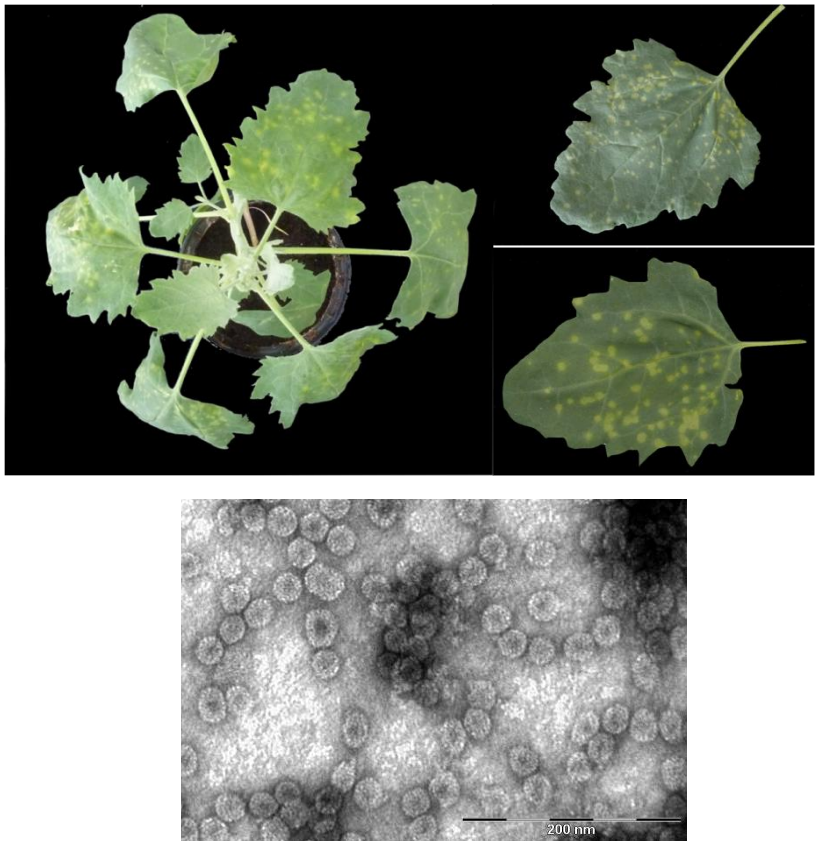



**Fig 4.3:** Chlorotic lesion on *C. quinoa* after 3 day post-inoculation (a) and viral particles with diameter of about 30 nm obtained from *C. quinoa* (b).

**Random-PCR amplification and sequencing**

Random amplification and sequencing of RNAs from purified virus allowed identification of short segments showing high degree of nucleotide identity with *Pelargonium zonate spot virus* isolated from Italian tomato (Finetti-Sialer & Gallitelli, 2003). In particular three sequences of 523 nts, 236 nts and 486 nts were obtained. The fragment of 523 nts showed 89% of nucleotide identity with PZSV RNA 1 (AJ272327) and both fragment of 236 nts and 486 nts showed 98% of nucleotide identity respectively against PZSV RNA 2 (AJ272328) and RNA3 (AJ272329). Having

regard to the high degree of nucleotide identity observed, primers for the subsequent characterization of the virus isolated from *A. chinensis* were designed on published sequences.

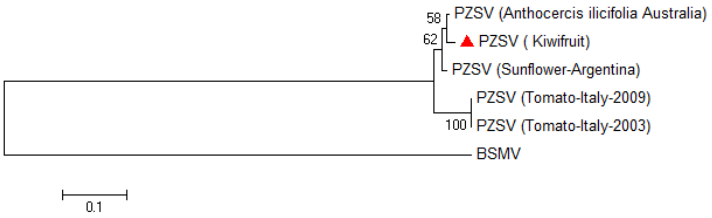
**Circular RT-PCR and full-length amplifications**

Circular RT- PCR for RNA 1 was performed with PZSV 123F – PSZV 1 330-349 R (Table 4.1) obtaining a 392 nts amplicon from which, primers for the full length amplification (PZSV RNA1endF – PZSV RNA1endR (Table 4.1) were designed. RNA-1 was 3,375 nts long, with a major single ORF 1a (2,946 nts) encoding a polypeptide which contains, in the N-terminal domain, conserved motifs of type I methyltransferases (aa 79-388) and in C-terminal conserved motif of the helicases of superfamily 1 (aa 691-959). RNA1 showed 91% nucleotide identity against RNA1 of Italian isolates from tomato (AJ272327) and the ORF1a showed nucleotide and amino acid identity respectively of 90 and 92% against the same isolate.

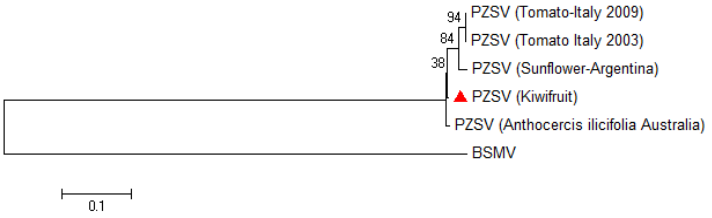
Amplifications of 5' and 3' ends of RNA2 was performed using primers PZSV 123F and PZSV2 213-233 R (Table 4.1) that produced a 230 nts fragment resulted 99% identical to the corresponding regions of PZSV RNA2 from tomato (AJ272328). Based on this sequence a new pair primers for amplification of the complete PZSV RNA 2 from actinidia were designed (PZSV RNA2endF - PZSV RNA2endR) (Table 4.1). The full length RNA 2 resulted of 2,435 nts in length, encoding for a polypeptide (ORF2, 2,141nt) showing high amino acid identity (99%) to Italian isolates from tomato.

Circular RT-PCR for RNA 3 was carried out using primers PSV 123F - PZS3 374-395R (Table 4.1) and an amplicon of 392 nts was obtained. Based on its sequence were designed primers for the full length amplification (PZSV RNA3endF - PZSV RNA3endR, Table 4.1). RNA 3 was 2,659 nts long and contains two ORFs, ORF 3a (930 nts) and ORF 3b (627nts), showing amino acid identity respectively of 98 and 100% against the Italian isolate from tomato (AJ272329).

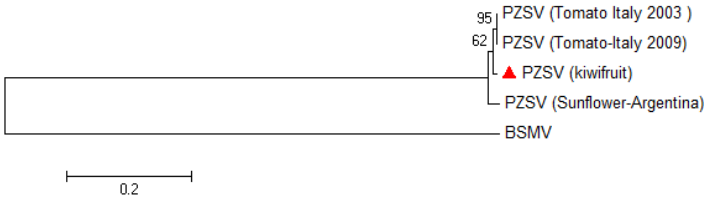
Phylogentic analyses was carried out comparing the single ORFs of PZSV from kiwifruit (Methyltranserase, Helicase, RNA-dependent RNA polymerase, Movement protein, Coat protein) with the single ORFs of other isolates of PZSV (Figure 4.4a-e) (sequences used in this study are listed in Table 4.2).



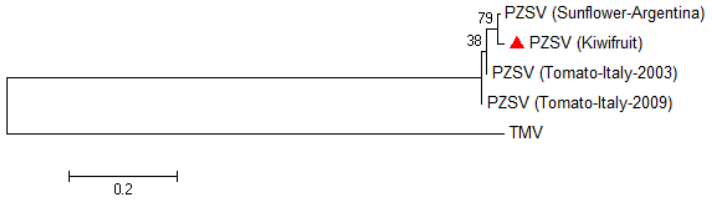

a) Methyltransferase



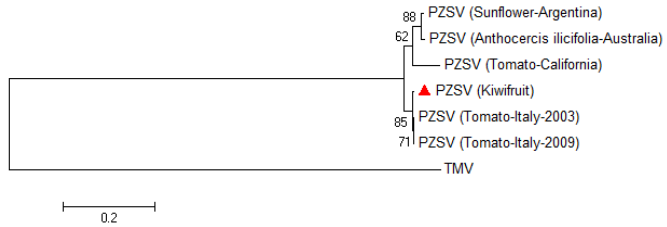

b) Helicase




c) RNA-dependent RNA polymerase




d) Movement protein




e) Coat protein

**Fig. 4.4:** Phylogenetic trees of amino acid sequences inferred by Minimum Evolution method of the (a) methyltransferase, (b) helicase, (c) RNA-dependent RNA polymerase, (d) movement protein and (e) coat protein of PZSV isolate from kiwifruit and other isolates. Bootstrap values are shown as percentages. *Barley stripe mosaic virus*(BSMV)(M16576; J04342) was used as the outgroup in trees (a), (b) and (c) and Tobacco mosaic virus (TMV) (AJ011933) in trees (d) and (e).

Phylogenetic analysis revealed that RdRp and CP proteins encoded by PZSV-Act isolate are strictly correlated with the protein domains of Italian PZSV isolated from tomato (Finetti-Sialer & Gallitelli, 2003) showing, respectively, aa identity of 99% 100 %. Notable exceptions are for the methyltransferase (MET), helicase (HEL) and MP proteins. In particular, MET protein from PZSV-Act isolate showed 98%, 97% and 92% aa identity against corresponding genes from Australian, Argentine and Italian isolates, respectively. Amino acid identity of HEL gene from PZSV-Act with Italian and Argentine isolates resulted of 96% and 97%, respectively, while MP aa sequence of kiwifruit isolate resulted more related to the corresponding sequence of sunflower isolate from Argentina (98%) than to the sequence from the Italian tomato isolate (96%).

**Table 4.2:** Abbreviations and Accession numbers of reference isolates used in this study.

| Strain | Accession Numbers | | |
|---|---|---|---|
| | **RNA1** | **RNA2** | **RNA3** |
| PZSV (Tomato; Italy 2003) | AJ272328 | AJ272329 | AJ272330 |
| PZSV (Sunflower; Argentina) | JQ350736 | JQ350739 | JQ350737 |
| PZSV (Anthocercis ilicifolia ; Australia) | KF790760 | / | / |
| PZSV (Tomato; Italy 2009) | NC003649 | NC003650 | NC003651 |
| PZSV (Tomato; California) | / | / | EU906913 |

**Serological and molecular detection**

PZSV from *A. chinensis* was detected successfully by ELISA test from symptomatic kiwifruit tissue using specific antisera raised against PZSV (ADGEN Phytodiagnostics, Scotland). Moreover, kiwifruit isolate of PZSV was detected by ELISA test also from all the herbaceous indicators mechanically inoculated with sap extract of PZSV-infected *A. chinensis* plants. Furthemore PZSV from *A. chinensis* was successfully detected from all infected plants also using the Dot blot DNA hybridization analysis.

All infected herbaceous plants and all symptomatic *A. chinensis* cv. Hort16A plants resulted positive also by RT-PCR assay, in particular also all obtained cuttings, tested

positive to PZSV only by molecular assay. The virus was not detected in any rootstock (*A. deliciosa* cv. Hayward) of symptomatic plants analyzed.

No serological or molecular reactions were observed using samples from healthy plants of either *A. chinensis* or herbaceous indicator species.

**Evaluation of RNA silencing suppressor activity of CP and MP proteins**

Patch test was performed on leaves of *N. benthamiana* 16C line by co-infiltration of pBIN61-GFP clone with pBIN61-CP or pBIN61-MP clones. Moreover the constructs pBIN61-p38, carrying the silencing suppressor protein p38 from *Turnip crinkle virus* (Deleris *et al*., 2006) and pBIN61-p0 from *Turnip yellows virus,* (Pfeffer *et al*., 2002) were used as a positive controls while the empty pBIN61 vector was employed as negative control.

The plants were observed 4 days post inoculation under UV light to evaluate the degree of fluorescence, therefore, the eventual silencing suppressor activity of the proteins assayed.

In plants agro-infiltrated with constructs pBIN61-MP or pBIN61-CP from PZSV no fluorescence was observed also 7 days after infiltration. Visible fluorescence was observed in positive controls, pBIN61-p38 and pBIN61-p0, but not in negative control (Figure 4.5).

**Fig 4.5:** Patch test on leaves of *N. benthamiana.* Visible fluorescence was observed in positive controls, pBIN61-p38 and pBIN61-p0 and not in negative control (empty pBIN61) and in pBIN61-MP or pBIN61-CP.

## Discussion

A new isolate of PZSV (PZSV-Act) has been identified in *A. chinensis* cv. Hort16A plants showing chlorotic and necrotic ring symptoms. This virus was first isolated from tomato (*Solanum lycopersicum*) in Italy and mentioned as *Tobacco streak virus* (Martelli and Cirulli 1969), it was later described to affect *Pelargonium zonale* plants showing concentric chlorotic rings in the leaves, from which it derived its name (Quacquarelli & Galitelli, 1979).

In this work, in contrast with previous reports exclusively from herbaceous hosts, PZSV has been detected, in Italy, in several symptomatic kiwifruit plants (*A. chinensis* cv. Hort16A), showing leaves symptoms and depressed areas on the fruits with consequently deformation of the berries. To our knowledge, this is the first detection of PZSV in a woody plants.

The virus can be successfully transmitted from *A. chinensis* to indicator plants, as *C. quinoa*, *N. benthamiana*, *N. glutinosa* and *N. tabacum*, by mechanical inoculation during spring but efficiency decrease during summer or fall. Furthermore cuttings obtained from symptomatic plants develop infected but symptomless leaves suggesting that the virus can be transmitted by propagation plant material but a long incubation period and also high viral titer, is necessary for symptoms induction. PZSV can be detected directly from symptomatic kiwifruit tissues by ELISA, Dot blot DNA hybridization and RT-PCR analyses. In addition the virus can be identified in symptomless plants only by RT-PCR (cuttings). Poor data are available regarding variability within PZSV isolates. High amino acid identity has been reported between Italian and Israeli tomato isolates (Lapidot *et al.,* 2010) and similar results have been obtained comparing the Italian isolates from tomato and kiwifruit (92% ORF 1a, 99%, 98% ORF2a, 98% ORF3a and 100% ORF 3b aa identity, respectively). Surprising phylogenetic analyses showed that only Coat protein and RNA-dependent RNA polymerase of PZSV detected in actinidia plants are closely related to the Italian isolates from tomato (Figure 4.4c and e). Methyltransferase, Helicase and Movement protein domains, in fact, showed highest identity against viral isolates from Australia or Argentina (Figure 4.4a, b and d) suggesting a possible origin of the PZSV-Act isolate different from Italian. On the other hands difference among amino acid sequence of ORF1a domains (MET and HEL) have been revealed between isolates from actinidia,

tomato, sunflower and *Anthocercis ilicifolia* also suggesting a relationship with host adaption.

In tomato PZSV is transmitted by seed, with efficiency of 29%, through pollen but infected pollen cannot horizontally transmit the virus to mother plants (Lapidot *et al*., 2010). No data are available about transmission from herbaceous host to kiwifruit and if transmission occurs naturally between kiwifruit plants, but it can be supposed that transmission occurs by pollen. New studies are therefore necessary to better investigate the biological and molecular behavior of PZSV that infect kiwifruit and its role as a casual agent of disease in *Actinidia* spp. With regard to the symptoms in the commercial orchard, PZSV is an important pathogen to manage.

No studies regarding Post-Transcriptional Gene Silencing (PTGS) suppression by PZSV encoded proteins have been reported, so far, but most viruses encode suppressors of gene silencing that indirectly regulate the level and speed of virus accumulation, frequently with effects on tissue invasion and disease pathology (Thomas *et al.,* 2003). According to our results the individual PZSV ORF, movement protein (MP) and coat protein (CP) (ORF 3a and ORF 3b, respectively) do not possess any activity of RNA silencing suppressor. We cannot exclude a possible interaction between MP and CP that can trigger the suppression of silencing then further experiment will evaluate the effect of co-infiltration, along with pBIN-GFP, of both constructs pBIN-MP and pBIN-CP. The effect of the mutual influence of CP and MP was in fact discharged from previous studies of *Tobacco mosaic virus* (TMV) (Conti *et al*., 2012) in which the two proteins suppress PTGS acting together. Another hypothesis is that the activity of suppressor gene silencing is carried out by a protein encoded by one of the two small ORF on the RNA1 and on RNA3 (Finetti - Sialer, 2003), or by two other ORF on the RNA2.

To date PZSV detected in Italy (this work) and CLRV detected in New Zealand (Blouin *et al.,* 2013) are the only viral agents associated with severe symptoms in kiwifruit plants. Both viruses are pollen transmitted in other hosts (Lapidot *et al.,* 2010; Card *et al*., 2007) and consequently have the potential to spread rapidly within individuals and between orchards, although pollen transmission in kiwifruit has not yet been proven.

It is clear that nursery practices and exchange of plant material play a key role in virus dissemination at both local and international level, therefore sanitary selection and, eventually, sanitation are the only functional means for producing certified stocks. For

PZSV there is a potential for transmission from reservoir herbaceous hosts to kiwifruit and subsequent spread by pollen or mechanical transmission that needs to be better investigated. Infected plants should be removed and equipment should be cleaned after use on infected vines and, more important, is necessary to verify and maintain the nuclear stock plants free from PZSV.

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# Chapter 5:

# Project Paper:

## Identification and characterization of two new viral species from *Actinidia chinensis*

# Identification and characterization of two new viral species form *Actinidia chinensis*.

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## Abstract

A new member of the family *Closteroviridae* was detected using 454 sequencing in *Actinidia chinensis* grown in Italy. The sequence of 18,848 nts contains one large open reading frame (ORF) carrying two papain-like leader proteases, the methyltransferase, the helicase and the RNA-dependent RNA polymerase. Additional ORFs contain the heat shock protein 70, the heat shock protein 90 and putative coat protein. The genomic organization and the phylogenetic analysis showed that this sequence is similar to that of members of the genus *Closterovirus*. Results were confirmed by polymerase chain reaction and traditional sequencing of amplicons and the 5' and 3' ends were determined by a RNA circular ligase and polyadenylation. A second viral RNA of 5,059 nts has been identified in the same plant and it shares high identity with members of the genus *Totivirus* and has been described as a new proposed species within this genus.

## Introduction

Kiwifruit (*Actinidia* spp.) has been domesticated only in the 20th century and since then it's became an important horticultural crop. The international kiwifruit production is concentrated in relatively few countries. The top four countries are China, Italy, New Zealand and Chile that collectively produce more than 80% of the world's kiwifruit crop (Belrose Inc., 2012). A range of virus families have been detected in *Actinidia* spp in Italy and New Zealand, actually between 2002 and 2013 a total of 13 different viruses has been identified (Blouin *et al*., 2013). Recent studies focused on survey of viruses infecting kiwifruit and two new detection have been described outside of New Zealand

and Italy, first in China where *Actinidia virus A* and *Actinidia virus B* were detected on *A. chinensis* (Zheng *et al.,* 2014) and subsequently in India with the characterization of an *Apple stem grooving virus* isolate infecting *A. deliciosa* (Bhardwaj *et al.,* 2014).

Our researches recently focused on plants of *A. chinensis* cv. Hort 16a showing chlorotic and necrotic rings on leaves followed by a general decline and death of the scion but not of the rootstock (*A. deliciosa* cv Hayward). In those plants several viruses were detected such as *Pelargonium zonate spot virus* (PZSV), *Actinidia virus A* (AcVA) and *Actinidia virus B* (AcVA) using a traditional generic methods such as electron microscopy or indicator plants as bioassays, to identify and characterize novel viral diseases (Biccheri *et al.,* 2015). We recently investigated the etiology dissection of this complex disorder by Next Generation Sequencing (NGS) approach, that has emerged as a powerful approach that provides rapid and exhaustive information about the infectious agents (viruses and viroids) present in plant tissue, especially since this method does not require prior knowledge about infecting viruses (Adams *et al.,* 2009; Kreuze *et al.,* 2009). By NGS, were detected two new viral agents sharing significant nucleotide and amino acid sequence identity one with members of the family *Closteroviridae* and the other with members of the family *Totiviridae*.

The family *Closteroviridae* is a group of related plant viruses that possess positive-stranded RNA elongated genomes (up to 20 kb) encapsidated in long, flexuous virions (650 - 2000 nm). Aphids, mealybugs and whiteflies are known to transmit these viruses (Agranovsky,1996; Karasev, 2000). The family is currently divided into four genera. Members of the genera *Closterovirus* and *Ampelovirus* have monopartite genome and are transmitted by aphids and mealybugs, respectively. Members of the genus *Velarivirus* have monopartite genome but, unlike other genera in the family, no insect vector has been associated with any of the members of this genus (Al Rwahnih *et al.*, 2012; Martelli *et al.*, 2012). Finally, members of the genus *Crinivirus* have multipartite genome and are transmitted by whiteflies (Karasev, 2000; Martelli *et al.,* 2002).

The family *Totiviridae* encompasses a broad range of viruses characterized by isometric virions, ranging from 30 to 40 nm in diameter, containing a not segmented dsRNA genomes coding, in most cases, only for a capsid protein (CP) and an RNA-dependent RNA polymerase (RdRp) (Ghabrial, 2008). The 28 species in the family *Totiviridae* are known to mediate mostly non cytopathic, persistent infection of diverse range of fungi

and protozoa. Five genera are currently recognized: *Giardiavirus*, *Leishmaniavirus*, *Trichomonasvirus*, *Totivirus* and *Victorivirus* (ICTV, 2014). Viruses infecting yeast, smut fungi, or filamentous fungi have been ascribed to the genera *Totivirus* and *Victorivirus,* while ones infecting parasitic protozoa in the other three genera (Ghabrial & Nibert, 2009; ICTV, 2014). In this paper we describe two new putative viral species: for the first, with a full genome sequence of 18,848 kb and showing less than 50% nucleotide sequence identity with other known viruses in the *Closteroviridae* family the name 'Actinidia latent virus' (AcLV) has proposed. The second putative virus has a full genome sequence of 5,059 kb, shows a genomic organization similar to members of the genus *Totivirus* and has been tentatively named Kiwifruit associated totivirus 1 (KaTV-1).

## Materials and Method

### Plants material

Plants of *A. chinensis* cv. Hort 16a, grafted on *A. deliciosa* cv Hayward, were collected from two orchards in Faenza province, Emilia-Romagna region, in May 2011. Scion of all plants resulted infected by PZSV, AcVA and AcVB while rootstocks were found infected by the two vitiviruses only (Biccheri *et al.,* 2015).

### Double-stranded RNA extraction

The samples was subjected to a double-stranded RNA (dsRNA) extraction as described by Valverde *et al.* (1990). The leaf material (1 g) was ground in liquid nitrogen, extracted with STE buffer (100 mM NaCl, 1 mM EDTA, 50 mM Tris pH 7.0) in the presence of bentonite, sodium dodecyl sulfate and TE-saturated phenol. Double stranded RNAs were then bounded to CF-11 cellulose in the presence of STE buffer containing 16.5% of ethanol, washed several times with the same buffer then eluted with STE, ethanol precipitated and resuspended in 50µl of nuclease-free water.

**Reverse transcriptase and amplification reactions**

Sample of dsRNA was subjected to RT followed by PCR as describe by Roossinck *et al.* (2010). Briefly, 1 µl of dsRNA was mixed with 7 µl of nuclease-free water , 1 µl of 10 mM TE (10 mM Tris 7.5, 10 mM EDTA) and 2 µl of primer 5'CCTTCGGATCCTCC $N_{12}$ 3' (20 µM). The samples were denatured at 99 °C for 2 min and subsequently chilled on ice. Eight µl of a mix containing 1 µl (5 units) of Superscript III (Invitrogen, Carlsband, CA, USA), 4 µl buffer 10X buffer (200 mM Tris-HCl, 500mM KCl), 2 µl dithiothreitol 0.1M and 1 µl dNTPs (10 mM each) was added to each sample. The samples were incubated on ice for 10 to 15 min before being placed at 50 °C for 1 h and subsequently 1 µl (1 µg) of ribonucleasi A (Sigma, prepared at 10 mg/ml in water) was added. Samples were incubated at room temperature for 15 min, heated at 85 °C for 2 min and purified with the 'QIAquick PCR purification' kit (Qiagen, Valencia, CA, USA) following the manufacture's protocol. Samples were amplified individually using 1.5 µl of the RT product in a 15 µl reaction. The reactions also contained a final concentration of 1 x PCR buffer 10x (20 mM Tris-HCl (pH 8.0), 40 mM NaCl, 2 mM Sodium Phosphate, 0.1 mM EDTA, 1 mM DTT) (Invitrogen, Carlsband, CA, USA), 170 µM dNTPs, 1 µM of Tag primer (5'ACGTAGATCGTACTACCTTCGGATCCTCC3'), 1,5 mM of $MgCl_2$ and 1 unit of Platinum® Taq DNA Polymerase (Invitrogen, Carlsband, CA, USA). The amplification program was: 94 °C for 1 min; 65 °C 0 s; 72 °C 45 s, with a slope of 9 (Ramp rate, °C/sec.), followed by 40 cycles of 94 °C 0 s; 45°C 0 s; 72 °C 30 s, with a slope of 5 (Ramp rate, °C/sec.) and a final 5 min at 72 °C and 5 min at 37 °C. Sequencing was performed in a 454 GS-FLX+ (Roche).

**Sequencing data analysis**

Reads from 454 pyrosequencing output were trimmed to remove sequences derived from the amplification primers. The resultant sequence data sets were processed for reads filtering and adaptor trimming using Geneious 7.0.6 (Kearse *et al*., 2012). In order to reduce the volume of NGS data reads were mapped to genomes reference of viruses previously detected on the plant, PZSV (AJ272327 (RNA-1), AJ272328 (RNA-2) and AJ272329 (RNA-3), AcVA (JN427014) and AcVB (JN427015) (Biccheri *et al.,* 2015). The remaining sequences were *de novo* assembled into larger contigs using Geneious

7.0.6 package and each contig obtained was screened for sequence homologies using both BLASTN and BLASTX against the GenBank database (http://www.ncbi.nlm.nih.gov/).

Based on the sequence of selected contigs, in order to fill gaps and confirm sequence obtained, specific primer pairs were designed to amplify fragments of about 2,500 bp subsequently cloned into pGEM-T easy vector (Promega, Madison, WI), according to manufacturer protocol and sequenced by classical Sanger method (MWG-Biotech AG, Germany).

NCBI conserved domain database (http://www.ncbi.nlm.nih.gov/) was used for computational analysis of the RNA genome sequence and the MEGA 6.0 package (Tamura *et al*., 2013) served to produce phylogenetic studies.

**Terminal regions**

The 5' and 3' termini were obtained by a Circular RT-PCR and poly(A) tailing respectively. For the 5' end a Circular RT-PCR was carried out following the Coutett's procedure with minor modifications (Coutett *et al.,* 1997). Fifteen µg of total RNAs were extracted with Spectrum™ Plant Total RNA kit (Sigma-Aldrich, Saint Louis, MO, USA) following the manufacturer's instructions then deccaped using 2.5 units of tobacco acids pyrophosphatase (TAP; Epicentre Technologies, Madison WI) in 20 µl of 50 mM sodium acetate pH 6.0, 1mM EDTA, 1% 2-mercaptoethanol, 0.1% Triton X-100 and incubated at 37°C for 1hr, no RNase inhibitor was added. After phenol-chloroform purification, the RNA was precipitated with ethanol and resuspended in 10 µl of nuclease-free water.

Circularization was performed for 3 hr at 37 °C in a total volume of 400 µl, 15 µg of deccaped RNA was incubated whit 20 units of T4 RNA ligase (Epicentre Technologies, Madison WI), 20 units of RNasin® Ribonuclease Inhibitor (40u/µl) (Promega, Madison, WI), 50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 20mM dithiothreitol, 100 µM ATP and 100 µg/ml acetylated BSA. After extraction with phenol-chloroform the RNA was precipitated with ethanol and resuspended in 10 µl of nuclease-free water.

Reverse transcription and PCR amplifications were performed using primers specifically designed on the sequence obtained. The RT was performed using 2 µl of

ligated RNAs, 1 µl of the specific reverse primer CLO-111circularR (10 mM) (Table S5.1 in Annex C) and 2 µl of nuclease-free water. Samples were placed at 70 °C for 5 min then immediately moved on ice. Fifteen µl of a mix containing 1 µl (5 units) of ImProm-II (Promega, Madison, WI), 4 µl of buffer 5x (250 mM Tris-HCl (pH 8.3 at 25°C), 375 mM KCl and 50 mM DTT), 1 µl of dithiothreitol 100 mM (Promega, Madison, WI), 1 µl dNTPs (10 mM each), 1.2 µl MgCl2 (25 mM) (Promega, Madison, WI) and 20 units of RNasin® (40u/µl) (Promega, Madison, WI). The RT program consisted in 25 °C for 5 min, 42 °C for 1 h and 70 °C for 15 min. The total volume of cDNA obtained was then adjusted to 50 µl by nuclease-free water.

The PCR amplification was performed in 25 µl of mix containing 5 µl of cDNA, 1 µl of the forward primer CLO 18,653F (10mM) (Table S5.1), 1 µl of the reverse primer CLO-111circularR (10mM) and 12.5 µl of GoTaq® Long PCR Master Mix 2X (Promega, Madison, WI). The PCR's program consisted in 94°C for 5 min followed by 35 cycles of 94°C for 15 sec, 58 °C for 15 sec, 72 °C for 1.5 min. The amplified DNA was analyzed in 1% agarose gel electrophoresis and stained with ethidium bromide. The amplicon obtained (327 nts), was excised from the gel, purified with Wizard® SV Gel PCR Clean-Up System kit (Promega, Madison, WI) according to the manufacturer's protocol, ligated into the pGEM-T easy vector (Promega, Madison, WI) which was subsequently used to transform competent cells of *Escherichia* coli strain M1022. Recombinant plasmids DNA from transformed cells was purified by by Wizard® Plus SV Minipreps DNA Purification System (Promega, Madison, WI) and sequenced by an external company (MWG-Biotech AG, Germany).

The determination of 3' end was carried out performing the poly(A) tailing as following: in a 20 µl total volume 10 µg of total RNAs were mixed with 1 µl of Poly(A)Polymerase (4 units) (Epicentre Technologies, Madison WI), 2 µl of Poly(A)Polymerase 10X reaction buffer (0.5M Tris-HCl pH 8, 2.5M NaCl and 0.1M $MgCl_2$), 2 µl of 10mM ATP and 20 units of RNasin® Ribonuclease Inhibitor (40u/µl) (Promega, Madison, WI), then incubated for 30 min at 37 °C.

The reaction was stopped by phenol-chloroform purification, the RNA was precipitated with ethanol and 40 µl of sodium acetate 3M pH 6.0 and resuspended in 10 µl of nuclease-free water.

Reverse transcriptase and PCR reactions were performed as described above using an oligo(dT) reverse primer and the forward primer CLO 17535-17559F (Table S5.1). The PCR's program consisted in 94°C for 5 min followed by 5 cycles of 94°C for 15 sec, 50 °C for 10 sec and 72 °C for 3 min and by other 30 cycles of 94°C for 15 sec, 58 °C for 10 sec, 72 °C for 3 min. An amplicon of 1,326 nts was obtained and was subsequently cloned and sequenced as above.

## Results

### Complete genome sequence of Actinidia latent virus

The dsRNA fraction extracted from leaves tissue of *A. chinensis* cv. Hort 16a plants was analyzed by 454 Life Sciences high throughput sequencing. This total genomic analysis produced 179,973 reads. BLAST analysis of the high quality reads against the GenBank database (Altschul *et al*., 1997) revealed 133,280 virus related sequences (74.05%). After filtering and mapping to genomes reference, 33,275 reads (ranging in length from 425 to 575 nts) were assembled by *de novo* assembly into a consensus sequence of 17,349 nts in length. Three different ORFs were initially identified on the sequence carrying conserved domains such as Helicase and Metiltransferase on ORF1a, Hsp70h and putative coat protein that showed significant similarity with corresponding domains of recognized or proposed members of the family *Closteroviridae,* in particular with the Persimmon virus B (variant 1, PeVBv1, acc.num. NC025967 and variant 2, PeVBv2, acc.num. AB923925; Ito, Sato and Suzaki, unpublished). Coverage of reads was not evenly distributed over the genome but highest in the region corresponding to the identified ORFs (Figure 5.1).

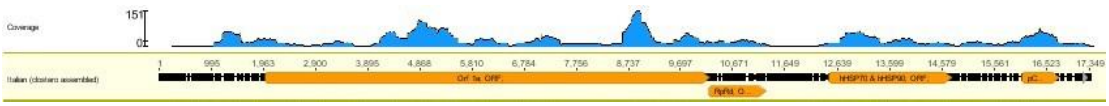


**Fig 5.1:** Sequence coverage, nucleotide positions and ORFs along the contig obtained by *de novo* assembly of 454 Life Sciences high throughput sequencing data set. The image was generated in Geneious pro 7.0.6 using the coverage feature.

To confirm genomic sequence of this putative novel member of the family *Closteroviridae*, 10 overlapping reverse transcription-polymerase chain reaction (RT-PCR) products were produced using 20 different primer pairs (Table S 5.1). High rate of correspondence resulted comparing the sequences obtained from Sanger method and sequence generated by *de novo* assembly as only 1,166 mismatches were reported, corresponding to the 6.6 % of the total nucleotides, including filling of 33 deletions which produced a new sequence of 17,382 nucleotides.

Finally circular RT-PCR and poly(A) tailing produced sequences of 327 and 1,326 nucleotides, respectively (see clone RB127 and RB128 in Annex D). Assembly of new sequences obtained resulted in the complete genome of AcLV which consists of 18,848 nucleotides.

**Computational analysis of Actinidia latent virus genome**

Computational analysis of the RNA genome sequence predicts 12 ORFs (Geneious 7.0.6) (Figure 5.2). Domains were identified using 'conserved domain database search' at NCBI (http://www.ncbi.nlm.nih.gov/). The first ORF (ORF1a) codes for a multifunctional 357-kDa (9,558 nts) protein that contains conserved domains for two papain-like leader proteases (interval 2,849-3,013 the first and 3,680-3,949 the second) with a predict molecular mass of 9.24 and 10.39 kDa, respectively. A methyltransferase domain is located from nucleotides 4,121 and 5,107 with a predict molecular mass of 33.04 kDa and a helicase domain from 8,666 to 9,487 nucleotides with a predict molecular mass of 31.31 kDa. The ORF1a showed amino acid identity of 18 % with ORF1a of PeVBv1 and of 17% with that of the *Mint virus 1* (MV-1, AY792620) within the genus *Closterovirus*. ORF1b is 1,533 nts, putatively encodes for RdRp with a predict molecular mass of 58.63 kDa and shares amino acid identity respectively of 42 % with RdRp of PeVBv2 and 38% with the RdRP of MV-1. Moreover, RdRp from AcLV showed amino acid identity of 49 % with the partial RdRp of *Olive leaf yellowing associated virus* (OLYaV, AJ440010), uncompleted and unassigned member of the family *Closteroviridae.*

Downstream of the polymerase three ORFs of 339, 675 and 159 nts in length, codify for three hypothetical proteins named p13, p25 and p6 with unknown functions and predict molecular mass of 13.56, 25.41 and 5.7 kDa, respectively. The small protein p6

contains high levels of hydrophobic amino acid (34/52aa) which could indicate a transmembrane domain. BLASTp searches revealed that p13, p25 and p6 have no significant similarity with viral proteins in the databases, but, instead, with proteins derived from various biological species (p13 is 29% aa identical to anion transport protein isoform X1 of *Oryctolagus cuniculus*, p25 is 27% aa identical to metal tolerance protein 4-like of *Setaria italic* and p6 is 41% identical to an hypothetical protein from *Henriciella marina*). The sixth ORF (1,755 nts) encodes for a heatshock protein 70 homolog (Hsp70h) (64.1 kDa) showing 32% amino acid identity with PsVBv2 and 28% with that of *Raspberry leaf mottle virus* (RLMV, NC008585) within the genus *Closterovirus*. In addition, Hsp70h from AcLV shares amino acid identity of 37% with that protein of OLYaV. The subsequent ORF (1,524 nts) produces a putative heatshock protein 90 (Hsp90h) with calculated molecular mass of 59.1 kDa and reveals 17% of amino acid identity with PeVBv2, 13% with the Hsp90h of the *Beet yellow virus* (BYV, NC001598) (genus *Closterovirus*) and 20% with the partial Hsp90h of OLYaV. ORF number eight encodes for a hypothetical protein of 29.87 kDa (p30) and is 728 nts in length from nucleotides 15,735 to 16,532. The following ORF (732 nts) contains the conserved domain for coat protein of *Closterovirus* with predict molecular mass of 27.38 kDa and showed amino acid identity of 22 % with the putative coat protein of PeVBv2 and 19% with the coat protein of RLMV. Downstream of the putative coat protein, three ORFs of 474, 213 and 405 nts, have been identified to encode for putative proteins with unknown functions showing no significant identity with known viral proteins. Those proteins, named p19, p8 and p15 have a predict molecular mass of 18.64, 7.57 and 15.49 kDa, respectively. Untranslated regions (UTRs) of the AcLV genome consist of 201 and 389 nucleotides respectively at the 5' and 3' ends.

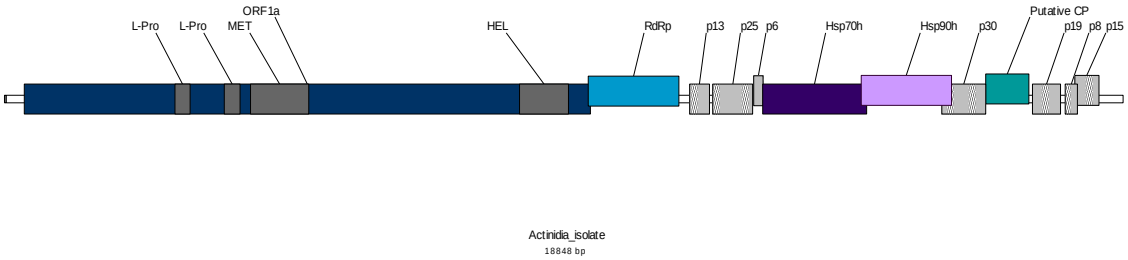


**Fig 5.2:** Schematic representation of the RNA genome of Actinidia latent virus (AcLV). The different segments represent ORF. L-Pro, leader protease; MET, methyltransferase domain; HEL, RNA helicase domain; RdRp, RNA-dependent RNA polymerase; Hsp70h, heatshock protein 70 homolog ; Hsp90h, heatshock protein 90 homolog; Putative CP, putative coat protein.

As in other *Closteroviridae*, conserved functional domains were separated by regions with variable aa content (Ghanem-Sabanadzovic *et al*., 2010).

Systematic sequence comparisons with the GenBank database revealed the highest nucleotide and amino acid sequence identity with members of the family *Closteroviridae.*

The Alignx Sequence Analysis tool of the Vector NTI Advance™ 11.5 package (Invitrogen Inc., Carlsbad, CA) revealed that all the identifiable protein domains encoded by the AcLV genome (ORF1a, RpRd, Hsp70h, Hsp90h and putative CP) show nucleotide and amino acid identity lower than 50% respect to orthologous domains of the other members of the family *Closteroviridae* (Table 5.1; virus abbreviations and accession number are described in Table S 5.2 Annex C).

Sequences comparison analysis regarding ORF1a encoded from AcLV, indicated 15-17% amino acid identity with ORF1a from the members of the *Closterovirus* genus *and* 15-18% of amino acid identity with ORF1a of unclassified sequences. The lower amino acid identity 12-16% was observed with ORF1a of members of the *Ampelovirus*, *Velarivirus* and *Crinivirus* genera. Concerning sequences comparison analysis of RdRp encoded by AcLV, this showed amino acid identity of 36-38% with RdRp of the members of *Closterovirus,* 37-42% with RdRp of the unclassified sequences and lower identity, ranging from 9 to 33%, with RdRp from members of the others three genera. The Hsp70h encoded by AcLV showed amino acid identity of 26-28% with Hsp70h from the members of the *Closterovirus* genus and 26-32% with hHSP70 of the unclassified sequences. Low amino acid identity (23-26%) has been reported with Hsp70h of the

members of the others three genera. The Hsp90h encoded by AcLV showed amino acid identity of 10-13% with Hsp90h of *Closterovirus* members, 10-17% with hHSP90 of the unclassified sequences and 8-12% with Hsp90h from members of the *Ampelovirus*, *Velarivirus* and *Crinivirus* genera. Sequences comparison analysis of pCP encoded from AcLV showed amino acid identity of 13-19% with pCP of the members of *Closterovirus* genus, 9-22% with pCP of the unclassified sequences *and* 10-17% with pCP of the other members of the family.

Moreover, no minor coat protein gene (mCP), has been detected within the genome sequence of AcLV similarly to *Pineapple mealybug wilt-associated virus 1* (PMWaV-1) of the genus *Ampelovirus*. The protein encoded upstream the putative CP p30 is, in fact, only 12 % identical to the putative CP and the p19, encoded downstream, is only 15 % identical to the putative CP (Figure 5.3). In contrast, the CP and CPm of other members of the family *Closteroviridae* have pronounced sequence similarity of 28-30%, primarily in their C-terminal regions as shown for *Beet yellow virus* and for *Pineapple mealybug wilt-associated virus 2* (PMWaV-2) (Figure 5.3)

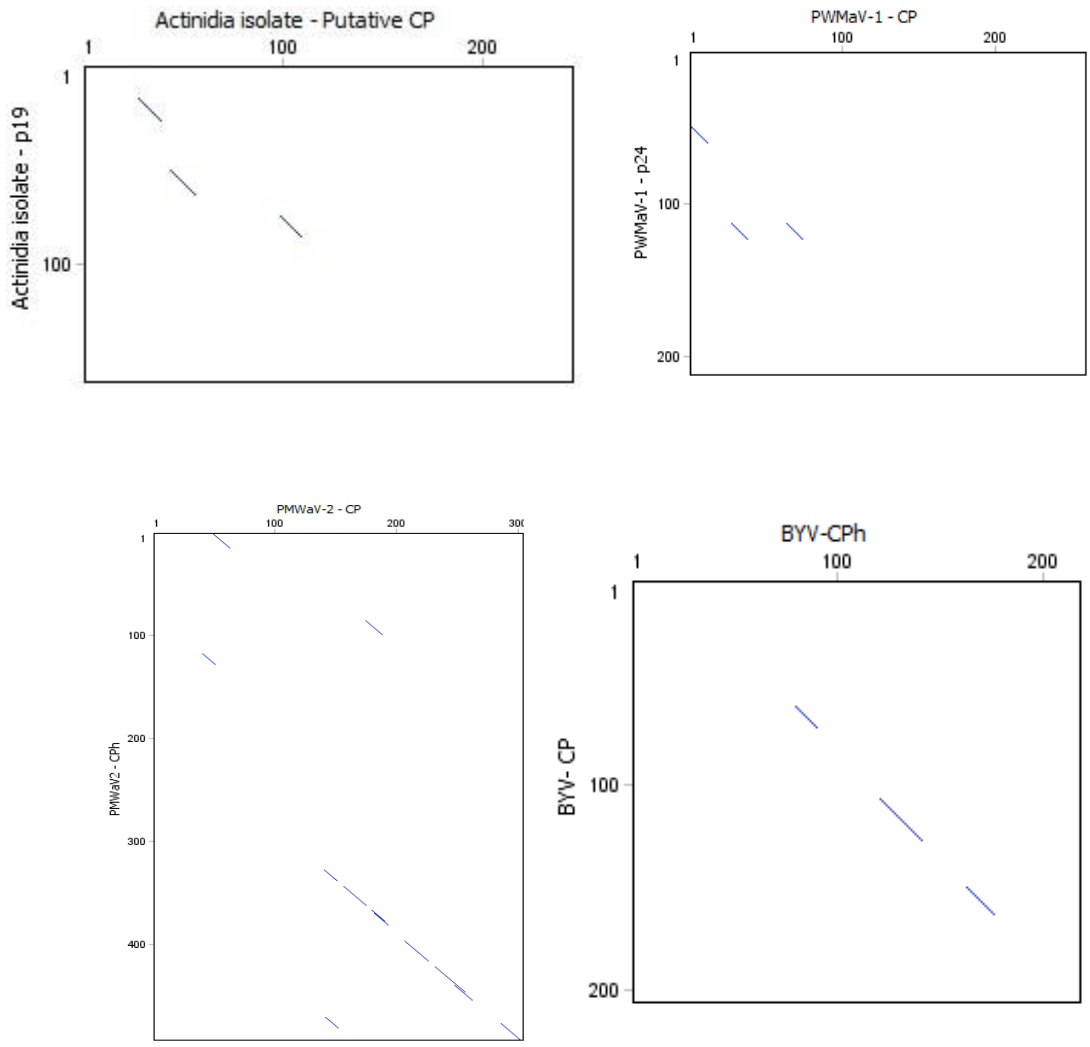


**Fig. 5.3**: Graphic representation of the low sequence similarity between the CP (*x-axis*) and p24 (*y-axis*) of AcLV (*top left*) and CP and p24 of PMWaV-1 (*top right*). In contrast, pronounced sequence similarity exists between the CP and CPh of PMWaV-2 (*bottom left*) and BYV (*bottom right*), particularly in the C-terminal regions of the proteins (*lower left corner* of graphs). Numbers on the axes correspond to amino acid residue positions for the proteins. *Graphs* were generated using the Dotblot in Geneious v7.0.6

**Table 5.1 :** Similarities (percentage) between amino acid (aa) sequences encoded by the genes in the AcLV genome (ORF1a, RdRp, Hsp70h, Hsp90h, pCP) and nucleotide (nt) and amino acid (aa) sequences encoded by the genus *Ampelovirus, Closterovirus, Velarivirus, Crinivirus*, unassigned and unclassified *Closteroviridae*.

**Actinidia latent virus** [a] Partial genome; [b] Partial sequences

| Genus | Virus | ORF1a | RdRp | Hsp70h | Hsp90h | pCP |
|---|---|---|---|---|---|---|
| | | % aa | % aa | %aa | %aa | %aa |
| *Ampelovirus* | GLRaV-1 | 14 | 28 | 24 | 10 | 15 |
| | GLRaV-3 | 13 | 9 | 25 | 8 | 13 |
| | GLRaV-4 | 12 | 9 | 23 | 13 | 15 |
| | LChV-2 | 16 | 11 | 23 | 13 | 13 |
| | PMWaV-1 | 13 | 24 | 25 | 11 | 12 |
| | PMWaV-2 | 15 | 28 | 25 | 7 | 15 |
| | PBNSPaV | 12 | 7 | 27 | 11 | 11 |
| *Closterovirus* | BYV | 16 | 37 | 26 | 13 | 16 |
| | CYLV | 16 | 37 | 26 | 10 | 15 |
| | CTV | 15 | 36 | 27 | 12 | 13 |
| | GLRaV-2 | 15 | 36 | 27 | 12 | 15 |
| | MV-1 | 17 | 38 | 26 | 10 | 17 |
| | RLMV | 15 | 36 | 28 | 13 | 19 |
| | SCFaV | 15 | 37 | 27 | 11 | 16 |
| *Velarivirus* | CoV-1 | 12 | 33 | 24 | 9 | 13 |
| | LChV-1 | 12 | 9 | 24 | 12 | 13 |
| | GLRav-7 | 13 | 29 | 26 | 10 | 12 |
| Unassigned | MVBaV | 16 | 31 | 24 | 10 | 13 |
| | OLYaV[a] | - | 49[b] | 37 | 20[b] | - |
| *Crinivirus* | BnYDV | 12 | 31 | 24 | 9 | 12 |
| | BPYV | 14 | 32 | 24 | 8 | 15 |
| | BYVaV | 14 | 33 | 24 | 9 | 15 |
| | CYSDV | 12 | 30 | 25 | 9 | 15 |
| | DVCV | 14 | 31 | 25 | 11 | 17 |
| | LCV | 13 | 31 | 24 | 9 | 12 |
| | LIYV | 13 | 11 | 26 | 9 | 16 |
| | SPaV | 14 | 7 | 25 | 10 | 15 |
| | ToCV | 12 | 32 | 25 | 10 | 10 |
| Unclassified | BVBaV | 14 | 26 | 24 | 9 | 15 |
| | GLRaV-5 | 13 | 26 | 23 | 12 | 14 |
| | GLRaV-6 | 12 | 9 | 23 | 12 | 14 |
| | GLRaV-9 | 12 | 24 | 23 | 12 | 16 |
| | GLRaV-10 | 12 | 25 | 23 | 11 | 15 |
| | GLRaCV | 12 | 10 | 24 | 11 | 16 |
| | GRSLaV | 15 | 38 | 27 | 11 | 17 |
| | Mint-Like V | 17 | 37 | 27 | 12 | 14 |
| | RLRaV | 16 | 36 | 27 | 10 | 18 |
| | BVA | 16 | 40 | 26 | 14 | 9 |
| | CYFV | 15 | 37 | 24 | 15 | 14 |
| | PeVBv1 | 18 | 41 | 31 | 16 | 21 |
| | PeVBv2 | 16 | 42 | 32 | 17 | 22 |
| | CCYV | 13 | 32 | 24 | 10 | 14 |

**Phylogenetic analyses of Actinidia latent virus**

To investigate the phylogenetic affinities between AcLV and viruses in the *Closteroviridae* family, multiple alignments of the complete amino acid sequences of the viral protein encoded by ORF1a, RpRd, Hsp70h, Hsp90h and pCP were generated and used to obtain phylogenetic trees comparing several members of the family having genome sequences available in GenBank (Table S5.2 in appendix C). Multiple sequence alignments were constructed using the default options of Clustal IW program and phylogenetic analyses were conducted using the minimum evolution methods using Poison model with 1000 bootstrap replicates from Molecular Evolutionary Genetic Analysis software (MEGA) version 6 package (Tamura *et al.* 2013). From phylogenetic relationship derived from the full length ORF1a, RpRd, Hsp70h, Hsp90h, pCP sequences it is observed that the proteins encoded from AcLV are always related to the branch including members of the genus *Closterovirus* but are also always closely related to proteins from unclassified viruses such as Blueberry virus A (BVA), PeVBv1 and v2 but also to the *Mint vein banding-associated virus* (MVBaV) (Figure 5.4 a, b, c, d and e). In particular sequences form AcLV, PeVBv1 and PeVBv2 always cluster in the same subgroup within the branch that include members of the genus *Closterovirus.* All Those viruses have the characteristics of a member of the family *Closteroviridae*, but the sequence analysis shows that they are distant from any characterized species of this family.

Based on the heat shock protein 70 (Hsp70h), the most conserved gene within the family *Closteroviridae*, PeVBv1 and v2 showed an amino acid identity in a range from 33 to 41 % and the closest relative (41% amino acid identity) was the *Cordyline virus 1* (CoV-1), a member of *Velarivirus* genus. Regarding BVA, the sequence analysis based on the Hsp70h resulted closest to the *Grapevine leafroll-associated virus 4* (GLRaV-4) (*Ampelovirus* genus) with 41 % amino acid identity. Concerning the AcLV, based on the complete amino acid sequence of ORF1a, RpRd, Hsp70h, Hsp90h and pCP the closest relative (18, 41, 31, 16, 22 % respectively) is the Persimmon virus B (PeVB, NC025967). The AcLV and PeVB showed a similar genomic organization, both lacking the minor Coat Protein genes (Table5.2).

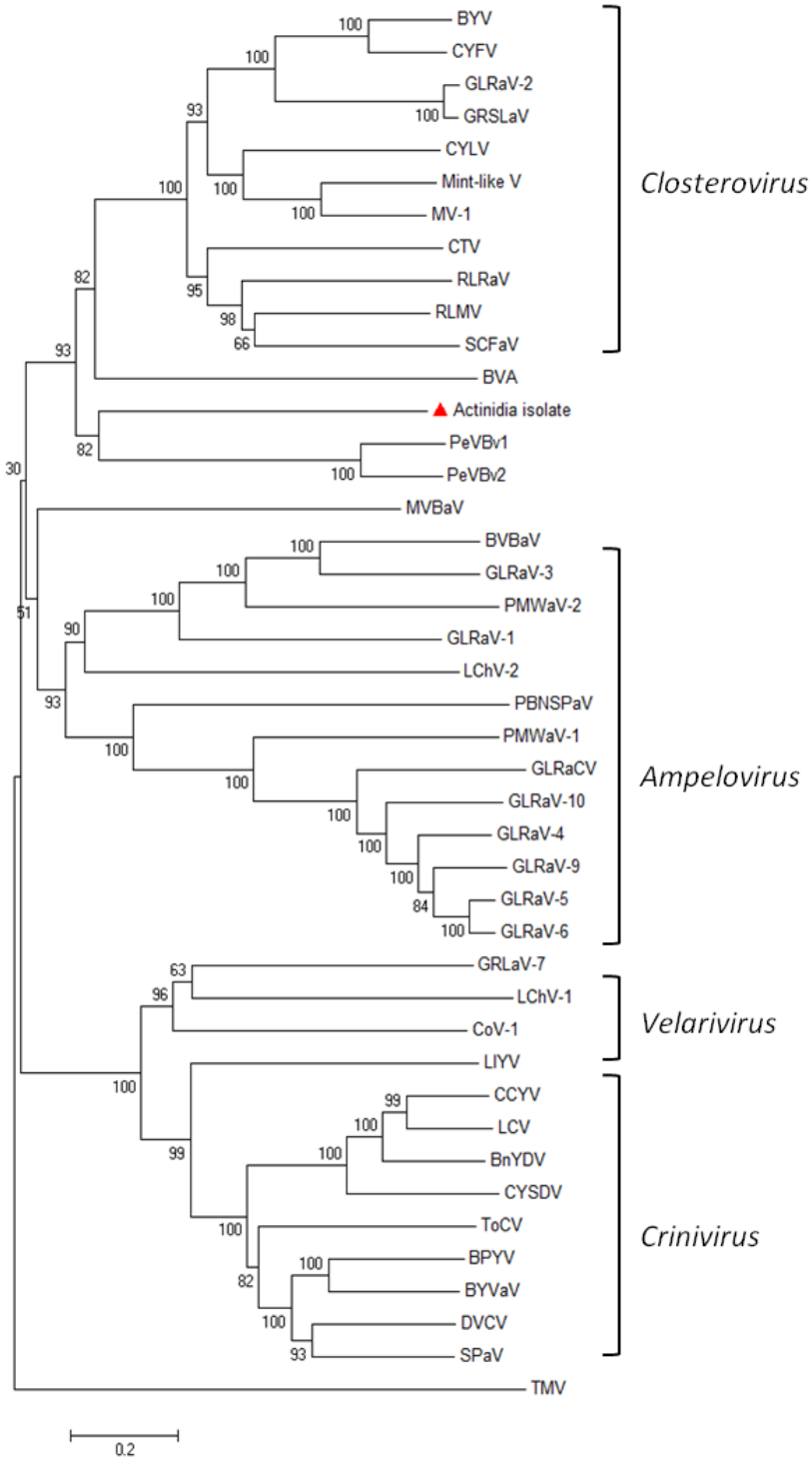


**Fig 5.4a:** Phylogenetic analysis of the ORF1a of AcLV and other species in *Closteroviridae* family. Bootstrap values are shown as percentages. ORF1a of *Tobacco mosaic virus* (TMV) (AF273221_1) was used as an outgroup.

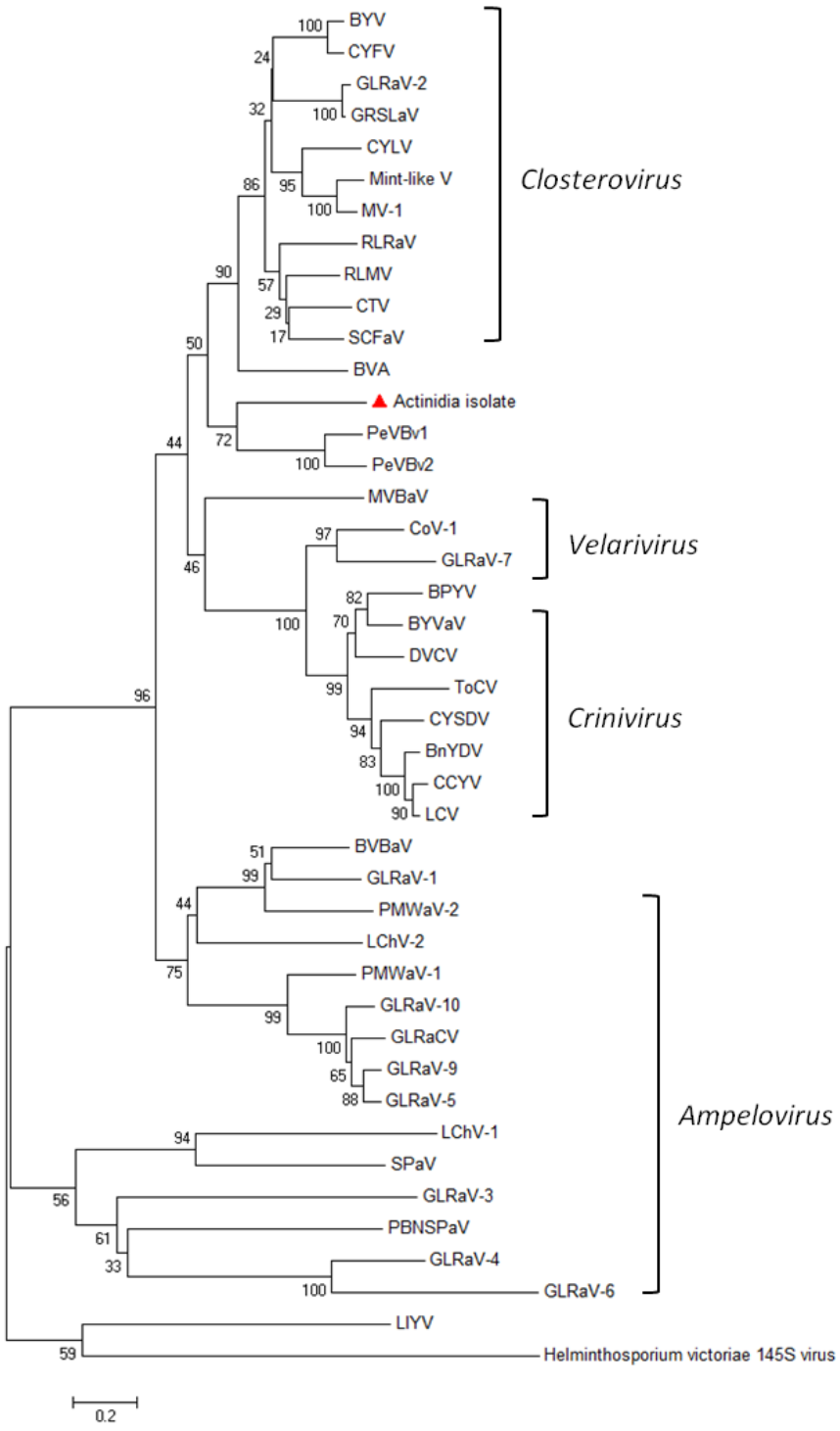


**Fig 5.4b:** Phylogenetic analysis of the RNA-dependent RNA polymerase of AcLV and other species in *Closteroviridae* family. Bootstrap values are shown as percentages. RdRP of *Helminthosporium victorriae 145S virus* (YP052858) was used as an outgroup.

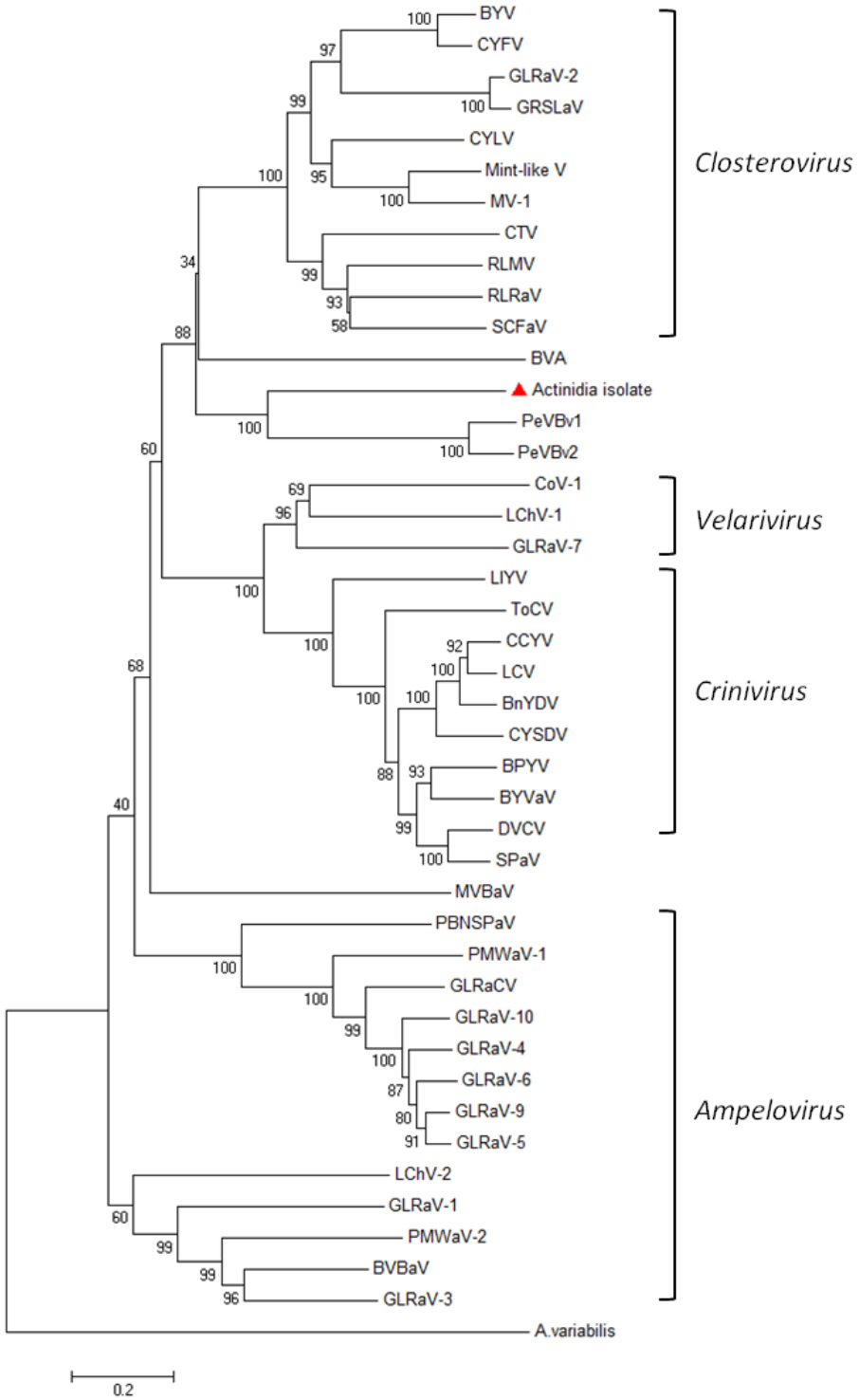


**Fig 5.4c:** Phylogenetic analysis of the heat shock protein 70 homolog sequences of AcLV and other species in *Closteroviridae* family. Bootstrap values are shown as percentages. Hsp70 of *Anabaena variabilis* (ABA20196) was used as an outgroup.

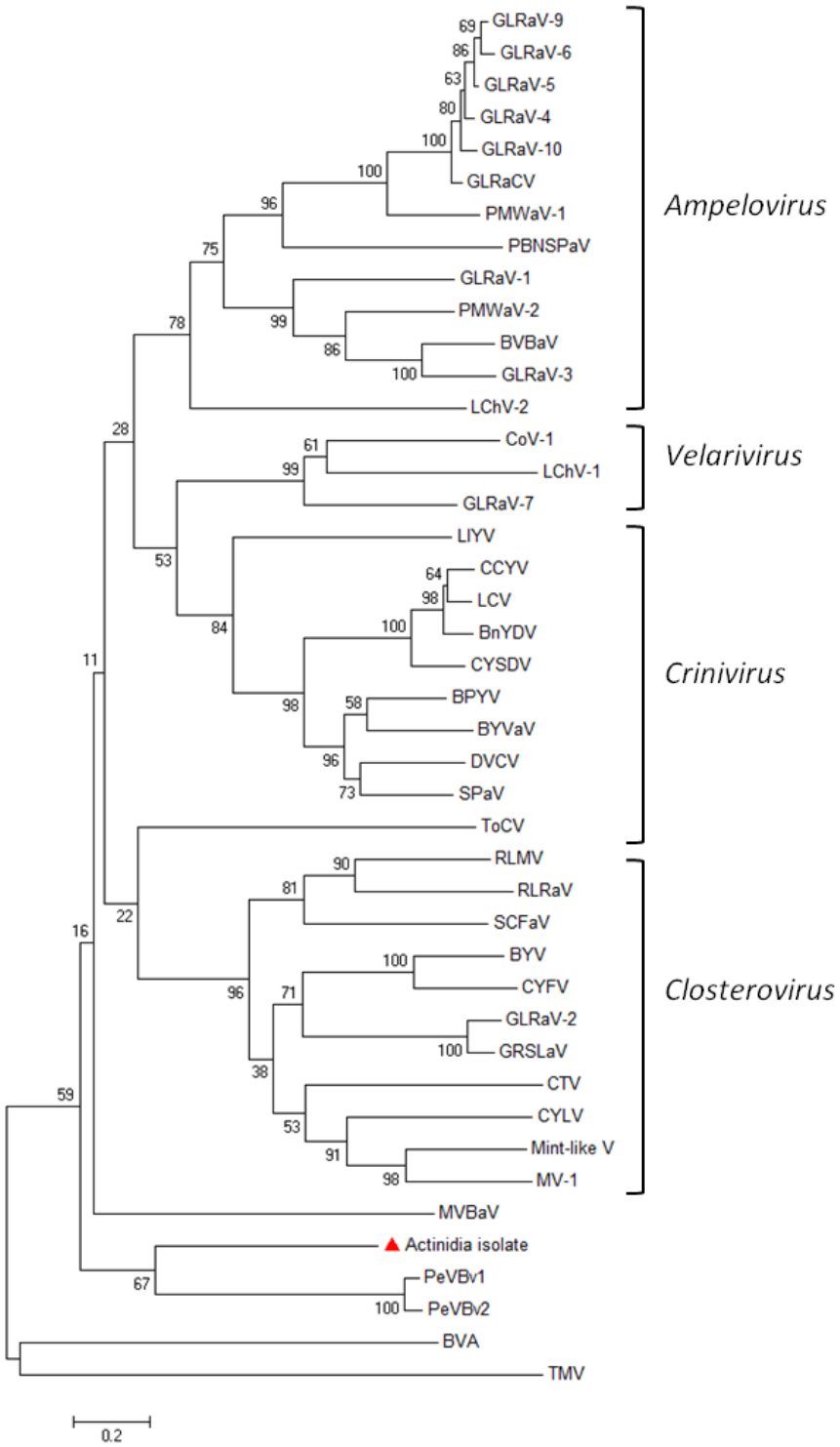


**Fig 5.4d:** Phylogenetic analysis of the putative Coat Protein sequences of AcLV and other species in *Closteroviridae* family. Bootstrap values are shown as percentages. CP of *Tobacco mosaic virus* (TMV) (P03576) was used as an outgroup.

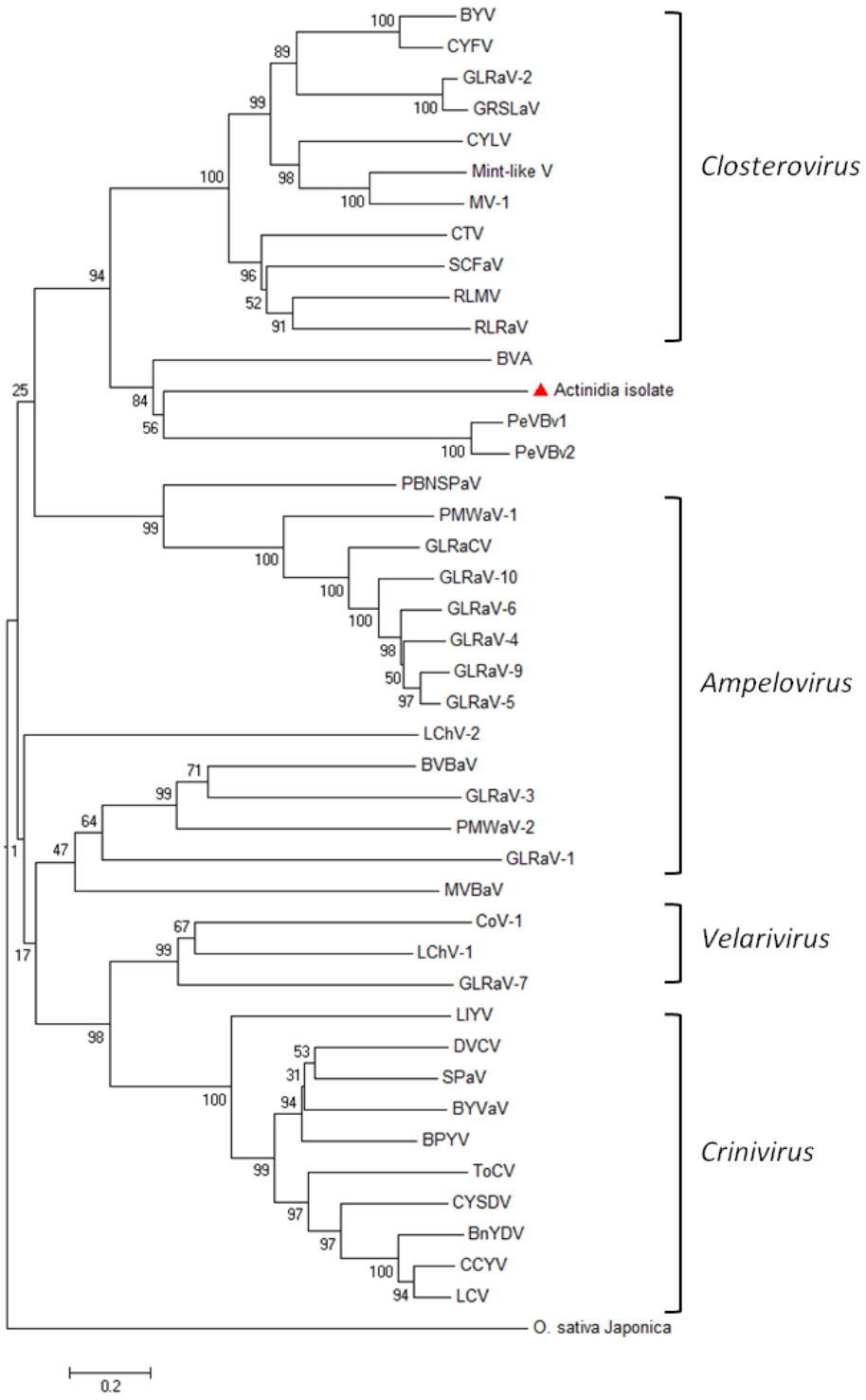


**Fig 5.4e:** Phylogenetic analysis of the heat shock protein 90 homolog sequences of AcLV and other species in Closteroviridae family. Bootstrap values are shown as percentages. Hsp90 of *Oryzia sativa* Japonica (BAD61715) was used as an outgroup.

**Table 5.2:** Comparison of AcLV, Pevbv1 and PeVBv2.

| Virus | Genome (nt) | 5' end (nt) | 3' end (nt) | N. ORFs | ORF1a nt/aa KDa | RdRp nt/aa KDa | Hsp70h nt/aa KDa | Hsp90h nt/aa KDa | pCP nt/aa KDa |
|---|---|---|---|---|---|---|---|---|---|
| AcLV | 18,848 | 291 | 389 | 12 | 9,558/3,185 356.58 | 1,533/510 58.63 | 1,755/584 64.1 | 1,524/507 59.06 | 732/243 27.38 |
| PeVBv1 | 18,569 | 182 | 282 | 12 | 9,633/3,149 351.28 | 1,548/515 59.58 | 1,758/585 65.05 | 1,512/503 59.0 | 810/269 30.27 |
| PeVBv2 | 18,030 | 184 | 235 | 12 | 8,967/2,988 332.74 | 1,548/515 59.18 | 1,758/585 65.05 | 1,512/503 59.0 | 810/269 30.27 |

**Characterization of Kiwifruit associated totivirus 1**

Sequence contig of almost 5,000 basepair, comprising approximately 2,800 reads, was generated by *de novo* assembly of data obtained from 454 Life Sciences high throughput sequencing analysis of dsRNA fraction extracted from leaves tissue of *A. chinensis* cv. Hort 16a plants. Full genome sequence of 5,059 nts was confirmed by RT-PCR, 5' and 3' RACE and overlapping cloning.

Homology search was done using BLASTN and BLASTX against the GenBank database and the sequence obtained showed the higher nucleotide and amino acids identity against members of the family *Totiviridae*. Computational analysis of the genome sequence predicts 2 single ORFs (Geneious 7.0.6) (Figure 5.5). The first ORF (from nucleotide 60 to 2,435) is 2,379 long nts and contains a conserved CP domain with a predicted molecular mass of 86.91 kDa. The second ORF (from nucleotide 2,504 to 5,026) resulted 2,523 nts in length and shows RdRp conserved domain with a predicted molecular mass of 95.89 kDa.

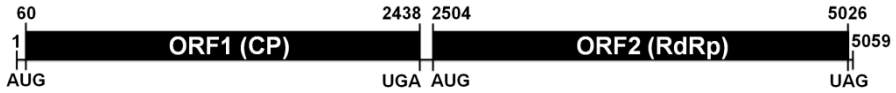


**Fig 5.5:** Genome organization of Kiwifruit associated totivirus 1. The dsRNA genome encompasses two large ORFs with the 5' ORF encoding a capsid protein (CP) and 3' ORF encoding an RNA-dependent RNA polymerase (RdRp)

The obtained CP and RdRp amino acid sequences were aligned with previously published sequences of the members of the family *Totiviridae* by the Alignx Sequence Analysis tool (Vector NTI Advance™ 11.5, Invitrogen Inc., Carlsbad, CA) and the amino acid identity (percentage) is listed in Table 5.3 (Accession numbers are reported in Table S5.4 in appendix C). Sequences of some proposed viral species such as Black raspberry virus F (BRVF, NC009890.1) have been also included in the analysis because of their high identity with the query sequence.

For RdRp, the amino acid identity between the kiwifruit isolate and the others members of the family *Totiviridae* is less than 50% but resulted of 53% against the sequence of BRFV. On the contrary the CP sequence showed an amino acid identity lower than 50% with all members of the family *Totiviridae* and only of 40% with the corresponded sequence of BRFV. In order to evaluate the evolutionary relationship between KaTV-1 and viruses in the *Totiviridae* family, multiple alignments were used for phylogenetic analyses by the minimum evolution method using Poison model with 1000 bootstrap replicates in the software MEGA version 6 (Figure 5.6a and 5.6b) (Tamura *et al*., 2013).

In both, CP (Figure 5.6a) and RdRp (Figure 5.6b) phylogenetic trees, it is possible to identify five distinct clades, each of which corresponding to the five genera *Victorivisus, Leishmaniavirus, Giardiavirus*, *Trichomonasvirus* and *Totivirus.* By CP and RdRp sequences the kiwifruit isolate results closest to Black raspberry virus F. The two viruses showed a similar genome organization and proteins proprieties (Table 5.4) but are clearly classified as a distinct viruses within the *Totivirus* genus.

**Table 5.3 :** Similarities (percentage) between amino acid (aa) sequences encoded by the genes in the Kiwifruit associated totivirus 1 genome and amino acid (aa) sequences encoded by the genus *Victorivirus*, *Leishmaniavirus*, *Giardiavirus*, *Totivirus* and unclassified sequences.

| | ***Kiwifruit associated totivirus 1*** | | |
|---|---|---|---|
| ***Genus*** | ***Virus*** | **RdRp** | **CP** |
| *Victorivirus* | *Aspergillus foetidus slow virus 1* | 12.9 | 8.1 |
| | *Helminthosporium victoriae virus 190S* | 13.4 | 6.8 |
| | *Coniothyrium minitans RNA virus* | 13.3 | 7.1 |
| | *Epichloe festucae virus 1* | 12.6 | 6.3 |
| | *Sphaeropsis sapinea RNA virus 2* | 12.8 | 6.5 |
| | *Gremmeniella abietina RNA virus L1* | 12.0 | 7.3 |
| | *Magnaporthe oryzae virus 2* | 13.6 | 7.5 |
| | *Magnaporthe oryzae virus 1* | 13.1 | 6.5 |
| | *Tolypocladium cylindrosporum virus 1* | 11.5 | 7 |
| | *Sphaeropsis sapinea RNA virus 1* | 12.9 | 7.2 |
| | *Beauveria bassiana RNA virus 1* | 12.2 | 8.3 |
| *Leishmaniavirus* | *Leishmania RNA virus 1 - 1* | 12.9 | 6.4 |
| | *Leishmania RNA virus 2 - 1* | 13.9 | 5.3 |
| *Trichomonasvirus* | *Trichomonas vaginalis virus 3* | 12.0 | 5.8 |
| | *Trichomonas vaginalis virus 4* | 11.1 | 5.9 |
| | *Trichomonas vaginalis virus 2* | 12.0 | 6.3 |
| | *Trichomonas vaginalis virus 1* | 13.8 | 5.8 |
| *Totivirus* | *Saccharomyces cerevisiae virus L-A L1* | 37.1 | 23 |
| | *Xanthophyllomyces dendrorhous virus L1A* | 28.4 | 10.1 |
| | *Tuber aestivum virus 1* | 36.0 | 23.8 |
| | *Xanthophyllomyces dendrorhous virus L1b* | 26.7 | 11.4 |
| *Giardiavirus* | *Giardia lamlia virus* | 16.0 | 16.0 |
| Unclassified | Gremmeniella abietina RNA virus L2 | 12.1 | 7.3 |
| | Eimeria brunetti RNA virus 1 | 12.3 | 7.3 |
| | Leishmania RNA virus 1 - 4 | 13.3 | 6.6 |
| | **Black raspberry virus F** | **53.0** | **40** |
| | Xanthophyllomyces dendrorhous virus L2 | 28.4 | 9.8 |
| | Botryotinia fuckeliana totivirus 1 | 12.5 | 6.3 |

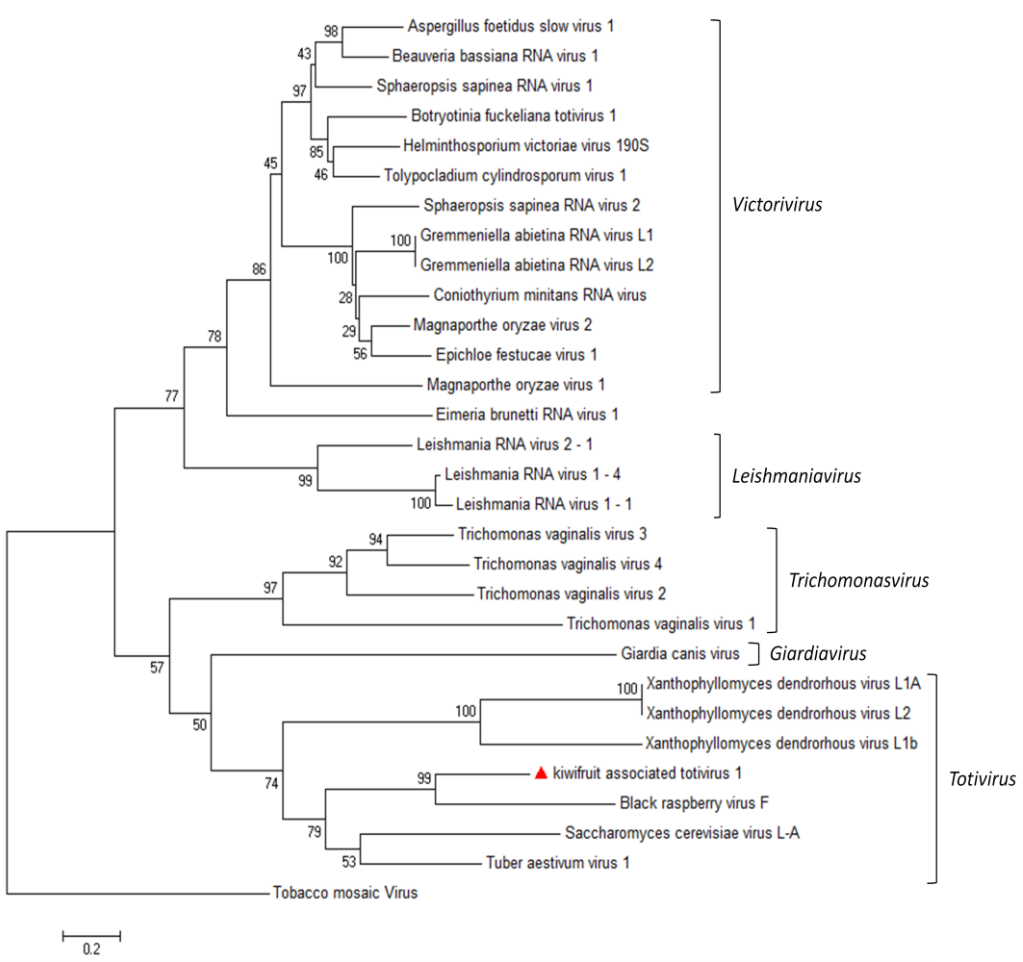


**Fig 5.6a:** Phylogenetic analysis of the Coat Protein sequences of the kiwifruit associated totivivirus 1 and other species in *Totiviridae* family. Bootstrap values are shown as percentages. CP of *Tobacco mosaic virus* (P03576) was used as an outgroup.

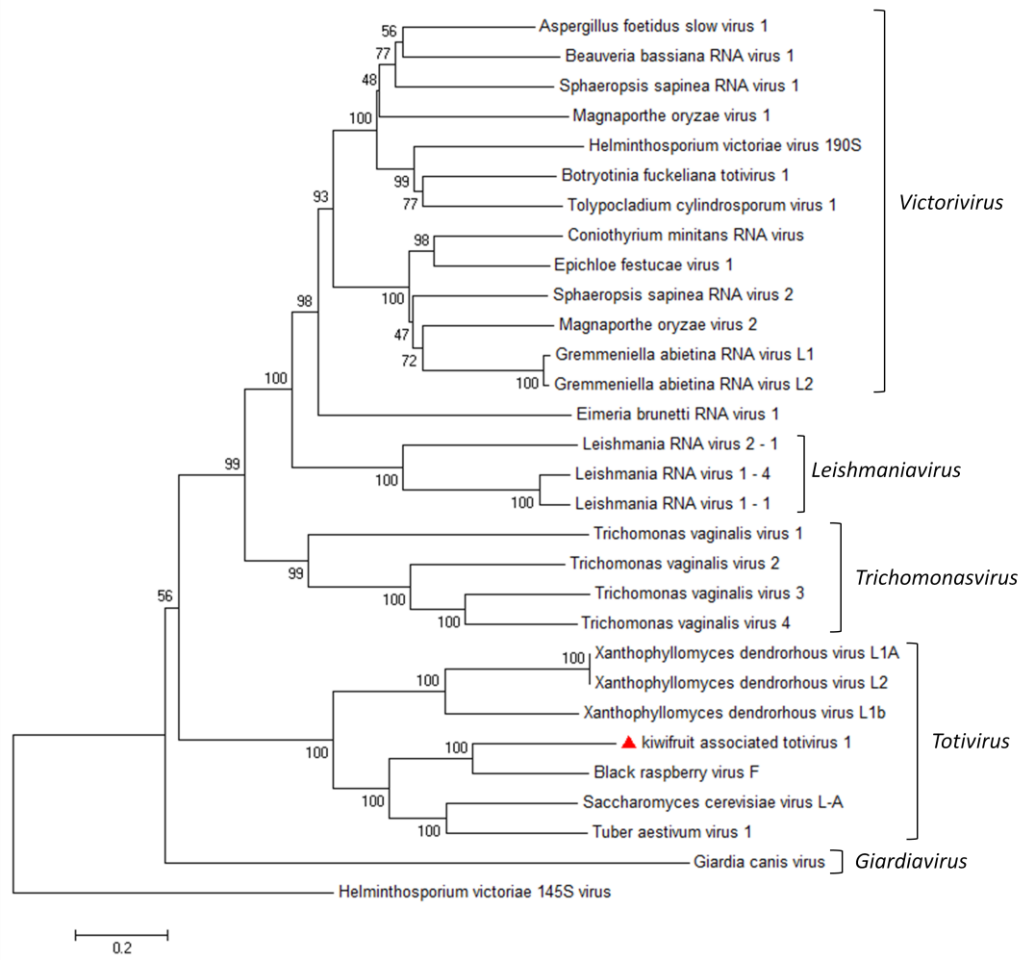


**Fig 5.6b:** Phylogenetic analysis of the RNA-dependent RNA polymerase of kiwifruit associated totivivirus 1 and other species in *Totiviridae* family. Bootstrap values are shown as percentages. RdRP of *Helminthosporium victoriae 145S virus* (YP052858) was used as an outgroup.

**Table 5.4**. Comparison of the KaTV-1 virus with the BRVF, the closest unclassified virus.

| Virus | Genome length (nt) | CP length nt-aa-kda | RdRp length nt-aa-kda |
|---|---|---|---|
| KaTV-1 | 5059 | 2379-792-86.91 | 2523-840-95.88 |
| BRVF | 5077 | 2310-769-85.28 | 2445-814-93 |

## Discussion

Our studies recently focused on plants of *A. chinensis* cv. Hort 16a showing chlorotic and necrotic rings on leaves followed by a general decline and death of the scion but not of the rootstock (*A. deliciosa* cv Hayward). *PZSV*, AcVA and AcVB have been detected in samples analyzed and, with the purpose to confirm the etiology of this complex disorder, the Next Generation Sequencing approach has been adopted.

As previously reported, also results described in this paper contribute to describe NGS as a powerful instruments to understand putative disorder in plant pathology. Unlike the traditional techniques, such as ELISA or PCR, this method requires no a *priori* knowledge of the suspected pathogen (Adams *et al.*, 2009). We have used the data from high-throughput sequencing to produce a census of the known viruses infecting diseased tissue and to pursue indications that a previously unknown virus may can have a role in the observed disease. Two new viruses were identified in the samples analyzed, a new putative members of the family *Closteroviridae*, Actinidia latent virus (AcLV) and a new putative member of the family *Totiviridae,* Kiwifruit associated totivirus 1 (KaTV-1). The AcLV has complete genome of 18,848 nts and encoded for twelve putative proteins. Its genome structure and organization (Figure 5.2) are similar to those of members of the *Closterovirus* genus (Figure 5.7). The RdRp of AcLV is probably expressed through a + 1 ribosomal frameshift as is presumed for other closteroviruses (Dolja *et.al,* 2006). In the overlapping ORF1a/ORF1b region of AcLV, the sequence GTGTTTGGCGTAATAAGGTCACAGGCTGTTCAAGATAG contained the start codon of ORF1b (GTG, underlined) and the stop codon of ORF1a (TAG, underlined). The sequence in red (GTGTTT) is identical to the sequence involved in the +1 ribosomal frameshift mechanism of other closteroviruses (Agranovski *et al*., 1994; Karasev *et al*., 1996; Zhu *et al.,* 1998). AcLV shares, according to sequences comparison analysis of all the identifiable protein domains encoded (ORF1a, RpRd, Hsp70h, Hsp90h and putative CP) nucleotide and amino acid identity lower than 50% with the orthologous domains of the other members of the family *Closteroviridae*. Higher amino acid identity of each ORFs, ranging from 16 to 42%, has been obtained with the unclassified Persimmon virus B (variant1 and variant 2) (NC025967; AB923925 respectively) (Table 5.1), a suggested member of the family *Closteroviridae.* AcLV and PeVB have the same genomic organization, in both viruses only five out of the twelve ORFs identified, encode for proteins associated with the *Closteroviridae* family, the others showed no significant identity with any of the known viral proteins. In addition, no minor coat protein (CPm) was identified in both AcLV and PeVB genomes. The CPm gene is thought to have originated from duplication and divergence of the CP gene then high amino acid identity ranging from 28 to 30% has been reported between the two genes within the viral genomes of BYV and PMWaV-2, respectively (Agranovsky *et al*., 1995).

In general, within closteroviruses, CPm gene has been identified upstream (*Grapevine leafroll-associated virus 2*) or downstream (*Mint virus 1*) the CP gene. On the AcLV genome, upstream and downstream genes of the CP include p30 and p19, respectively (Figure 5.2). The p29 protein is only 12 % identical to the AcLV CP and no significant amino acid sequence similarity has been reported within their C-terminal regions. This is in contrast with previous reports that indicate CP and CPm of most of the members of the family *Closteroviridae* to have pronounced sequence similarity, primarily in their C-terminal regions (Figure 5.3). Same result was reported for P*ineapple mealybug wilt-associated virus 1* and *Grapevine leafroll-associated virus-Pr* members of the *Ampelovirus* genus and the unassigned Blueberry virus A (Maliogka *et al*. 2009; Melzer *et al*. 2008; Isogai *et al.,* 2013). Moreover, protein p30 shows 31% of amino acid identity with p21 of OLYaV (AJ440010), uncompleted and unassigned *Closteroviridae* and 26 % of amino acid identity to p30 of PeVB (AB923925). The p19, located downstream of the AcLV CP and that lacks significant similarity to other plant virus proteins, is only 15 % identical to the AcLV CP and no significant similarity has been found between the C-terminal region the two proteins. Thus, the p30 and p19 do not appear to be a paralog of the AcLV CP as the similar regions between the CP and p30 and CP and p19 genes are scattered throughout the alignment, in contrast to those of other *Closteroviridae* members, where the CP and CPm display higher similarities in their C-terminal regions connected throughout the alignment (Figure 5.3).

The species demarcation criteria for closteroviruses includes the amino acid sequence of relevant gene products (CP, CPm and HSP70h) differing each by more than 25 % (ICTV, 2013). The CP and Hsp70h proteins encoded by the AcLV genome shared highest identities with CP and Hsp70h genes of 28 and 19 %, respectively, within the family *Closterovirus* and of 22% and 31 %, respectively, with corresponding genes of PeVB. The distinguishing properties of the genera in the family *Closteroviridae* are listed in the Table 5.5. According to these criteria, AcLV is in good agreement with the proprieties of member of the genus *Closterovirus*. In addition, Table 5.1 shows that the amino acid sequences of the AcLV ORF1a, RdRp, Hsp70h, Hsp90h and CP shared the highest amino acid sequence identities with those of closteroviruses. The phylogenetic analysis of the ORF1a, RdRp, HSP70h, HSP90h and CP inferred using the minimum evolution method indicated that AcLV could be a new member of genus C*losterovirus*

(Figure 5.4a-e). The viruses of the genera *Closterovirus*, *Crinivirus* and *Ampelovirus* are predominantly transmitted by aphids, whiteflies and mealybugs, respectively (Fauquet *et al. 2005*). Karasev (*2000*) suggested a correlation between the RdRp phylogeny and the type of insect vector. The RdRp phylogeny places AcLV closer to the genus *Closterovirus* than to the genera *Crinivirus*, *Ampelovirus* and *Velarivirus* (Figure 5.4b) then suggests its transmission by aphids but, to date, this aspect has not been investigated in kiwifruit. AcLV has been identified in symptomatic plants as a member of a mixed infection and seems able to infect the scion (*A. chinensis* cv. Hort16A) but also the rootstock (*A. deliciosa* cv Hayward). In this plant the vitiviruses AcVA and AcVB were also detected (Biccheri *et al*., 2015). The new putative *Closterovirus* could represent the helper virus that may potentially assist the natural transmission of Actinidia vitiviruses. Vitiviruses are often detected in co-infections with a member from the family *Closteroviridae*. In grapevine, *Grapevine leafroll associated virus 1* (GLRaV-1 genus *Ampelovirus*) has been reported to be co-transmitted with the vitivirus *Grapevine virus A* (Hommay *et al.*, 2008).

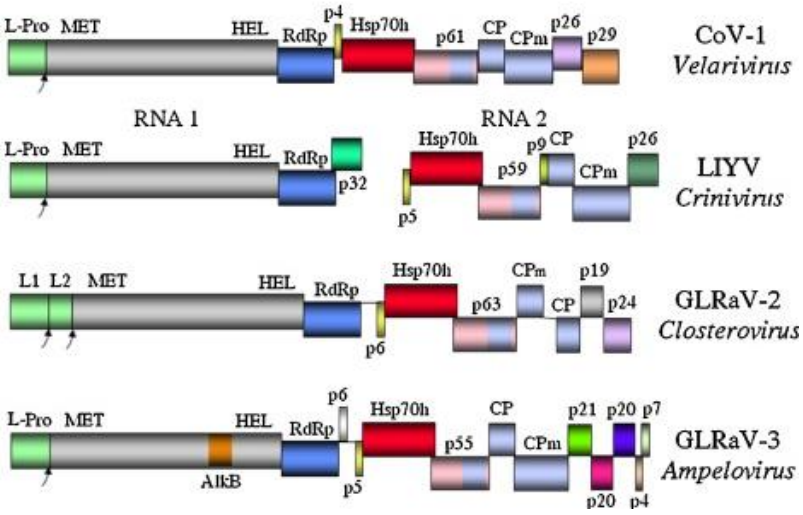


**Fig 5.7:** Genome organizations of genus within the family *Closteroviridae.* The different segments represent ORFs; their vertical heights represent the different frame registers. Shared colors represent conserved sequences. L-Pro, leader protease; MET, methyltransferase domain; HEL, RNA helicase domain; RdRp, RNA-dependent RNA polymerase; HSP70h, heat shock protein 70 homolog; CP, coat protein; CPm, minor coat protein. (*Source:* Al Rwahnih *et al*, 2012).

**Table 5.5:** Distinguishing properties of the genera in the family *Closteroviridae* compared with AcLV

| Genus | Virion Length (nm) | Rna species (No.) | Genome size (kb) | ORF (No.) | Replicasi (kDa) | HSP70h (kDa) | Cp (kDa) | Cpm (kDa) | Vector |
|---|---|---|---|---|---|---|---|---|---|
| AcLV | unknown | 1 | 18.848 | 12 | 356.58 | 64.1 | 27.38 | abs | unknown |
| Closterovirus | 1350-2000 | 1 | 14.5-19.3 | 8-12 | 349-367 | 65-67 | 22-25 | 24-27 | Aphid |
| Ampelovirus | 1400-2000 | 1 | 13.0-18.5 | 7-12 | 245-293 | 57-59 | 28-36 | 50-56 Abs | Mealybugs |
| Crinivirus | 750-900 | 2 or 3 | 15.6-17.9 | 9-13 | 267-280 | 62-65 | 28-29 | 53-80 | Whiteflies |
| Velarivirus | 1500-1700 | 1 | 16.4-16.9 | 8-9 | 258-270 | 62-70 | 34-36 | 69-77 | no known vector |

To complete the viral etiology of the symptomatic plants analised in the present study, the complete genome of KaTV-1 has been described. The two encoded proteins, CP and RdRP showed amino acid identity of 23 and 37%, respectively whit CP and RdRp of *Saccharomyces cerevisiae virus L-A L1,* member of the genus *Totivirus*. Moreover KaTV-1 share aminoacid identity of 40 and 53 % respectively whit CP and the RdRP encoded by the unclassified Black raspberry virus F. In addition, pairwise sequence comparisons of the deduced amino acid sequences encoded by KaTV-1 (Table 5.3) as well as phylogentic analysis indicated that it is closely related to the totiviruses.

According to the species demarcation criteria of the family *Totiviridae*, less than 50% sequence identity at the protein level generally reflects a species difference in particular if the viral agent is found only in distinct host species (ICTV, 2014). According to these criteria, KaTV-1 is proposed as a new member of the genus *Totivirus* within the *Totiviridae* family.

Totiviruses are often associated with latent infections of their fungal hosts but recently some authors speculated on their ability to replicate within plant cells (Roossinck, 2013). More investigations will be necessary in order to search for an eventual fungal host on the *A. chinensis* plants analyzed and to clarify the role, if any, of KaTV-1 on infected plants.

The multivirus infection revealed by deep sequencing in kiwifruit plants, in fact, may reflect a complex interaction within the different players that need the understanding of diversity and synergies within the host and viral species that can infect it.

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# Chapter 6:

## General discussion and conclusion

## General discussion

Kiwifruit crop is relatively disease free in many countries, this does not appear to be the situation in China, where the genus originates (Lin & Gao, 1995). The apparent freedom from disease in other areas may be because production has frequently been initiated using imported seed, which probably carries fewer diseases than nursery stock. However, there is increasing commercial pressure to transfer genetic material between countries in the form of woody cuttings, for example for breeding purposes. Such material has advantages over seed as the precise genetic profile of the material is known. However, the phytosanitary risks associated with cuttings are likely to be significantly greater than those associated with seeds. An example of this increased risk occurred during February 2001, when cuttings of *A. chinensis*, exported from China, were detained in post-entry quarantine in New Zealand because they exhibited unusual disease symptoms. It was subsequently confirmed that the plants were infected with *Apple stem grooving virus* (ASGV) (Clover *et al.,* 2003).

To date, thirteen different viruses have been isolated from *Actinidia* spp. in Italy and New Zealand from only four laboratories, three in New Zealand and our lab in Italy (DipSA, University of Bologna) and all have been identified in kiwifruit over the past decade. The increased interest in disease-resistant cultivars of kiwifruit as well as the recent discovery of pathogenic viruses should stimulate further research.

New technologies such as high-throughput sequencing is likely to detect additional viruses, including some that are not mechanically transmissible, especially since this method does not require prior knowledge about infecting viruses. Examples of this is a new members of the families *Closteroviridae* and *Totiviridae* detected by 454 sequencing.

Many of the viruses described until now are not associated with important symptoms and/or spread (the non-specialists) and are not considered to be economically important in commercial orchards.

To date, only two disease-inducing viruses with localised spread and severe symptoms have been reported, *Cherry leaf roll virus* (CLRV) in New Zealand and *Pelargonium zonate spot virus* (PZSV) in Italy (Biccheri *et al.,* 2015). Although the two viruses were identified recently, they have already been associated with significant symptoms, with

consequences for yield. It is too early to assess the spread of these virus infections, but they are being monitored as there is a potential for transmission from reservoir hosts to kiwifruit and subsequent spread by pollen or mechanical transmission that needs to be better investigated.

Of these 13 viruses detected from kiwifruit, five (*Actinidia virus A* (AcVA), *Actinidia virus B* (AcVB), *Actinidia virus X* (AVX), Actinidia citivirus and the novel putative *Closterovirus* Actinidia latent virus (AcLV), to date, have not been isolated from another host. With the exception of AVX, these are putatively kiwifruit specialists, as they are related to viruses that have a narrow host range. These kiwifruit-specialist viruses mostly cause leaf symptoms, but can also be latent. However, since these viruses have only been studied in non-commercial orchards, their effect on yield and plant longevity is unknown. No vector of these viruses has been identified yet and no movement to new kiwifruit plants has been observed except by grafting. Since it is likely that these viruses originate from wild kiwifruit populations in Asia, insect vectors are probably present in these countries. These specialist (or host adapted) viruses can also infect plants without symptoms and would be easily overlooked and propagated within nurseries or orchards. The specialist viruses might pose a risk to kiwifruit growing within new environments if they infect new cultivars, interact with other viruses, or if a vector is present.

The identification of these 13 viruses that can infect kiwifruit has important repercussions for orchard management, especially for nurseries that propagate kiwifruit. It is important to have nuclear stock plants that are free from known viruses. These health precautions should preclude the chance of infection from the specialist group of viruses.

Kiwifruit breeding has a remarkable depth of genetic variation to exploit for new commercial attributes such as flavours, colours and nutritional benefits (Ferguson and Huang, 2007). The germplasm should also be screened for those vines that are either resistant or tolerant to each of these 13 viruses. The range of symptoms observed to date for some individual viruses on different cultivars suggests that there is a potential virus resistance or tolerance. To date the industry has been fortunate to have selected a cultivar, *A. deliciosa* cv. Hayward, that has shown very good resistance or tolerance to disease in general and viruses in particular. Currently there is a need for tolerance to

the bacterium *P. syringae* pv. *actinidiae* but screening for virus tolerance would also be prudent. Viruses are common in most crop species, particularly those that are vegetatively propagated, and the effects of viruses on crops can range from insignificant (e.g. latent viruses) to severe impacts on crop production (e.g. *Citrus tristeza virus* in *Citrus* species and *Plum pox virus* in *Prunus* species) (Bar-Joseph *et al*., 1989; Levy *et al*., 2000; Pearson *et al*., 2007). The effects of virus infection often differ between host species or cultivars, have some impact on growth, yield or quality of crops, and may be exacerbated by the accumulation of multiple viruses and/or the selection of more aggressive strains (Pearson *et al.,* 2011; Wang & Valkonen, 2008). It is therefore vital to eliminate known viruses from germplasm collections to prevent the spread of viruses through propagative material in order to minimise the possibility of serious viral disease in kiwifruit in the future (Pearson *et al.,* 2007). The use of virus-free propagation material is one of the most effective methods of controlling virus diseases of vegetatively propagated plants (Lozoya-Saldaña & Dawson, 1982).

A range of virus families have been detected in *Actinidia* spp and it is probable that other viruses will be detected over the next decade, particularly as researchers start actively looking for viruses in other major kiwifruit growing countries as China and Chile. Moreover with technologies as NGS, will be useful to identify more RNA and DNA viruses and/or viroids, and to pinpoint rapidly the cause of disease when present, but could also uncover latent viruses and potentially viruses that are beneficial (Roossinck, 2011). A major challenge is to undertake the basic research on the virus ecology so that vectors, host range and impacts on the plant host can be characterized.

**Do viruses pose a threat to kiwifruit?**

Although there have not been any reports of major virus epidemics in kiwifruit. Based on experience with virtually all other woody crops, unless preventative measures are taken, it is probably only a matter of time before there is a significant viral epidemic in kiwifruit. Consequently it is important to compile as much information as possible on the viruses able to infect kiwifruit and put in place procedures to prevent the international spread of viruses in *Actinidia* germplasm and the distribution of infected bud wood through virus testing and high health schemes.

We have transmitted viruses from kiwifruit to a number of herbaceous indicator plants, but transmission of these isolates in indicators back to kiwifruit has been more difficult. For example, attempts to transmit AcVA and AcVB from *N. occidentalis* to *A. chinensis* by mechanical inoculation were unsuccessful for AcVA and although AcVB gave symptoms on leaves acropetal to the inoculated leaves, the infections did not persist (M. Pearson, personal communication).

While it is now clear that Actinidia is susceptible to a wide range of viruses we have noted that *A. chinensis* is more susceptible to virus infection than *A. deliciosa.* Since most commercial plantings of kiwifruit are *A. deliciosa,* this might explain why it took seventy years from the initial commercialisation of kiwifruit to the first definitive identification of a virus in kiwifruit in 2003.

**Mitigating virus spread**

Mitigation of virus spread is necessary both international and a local levels. Evidence of the introduction of viruses in scionwood imported from China to Italy has been demonstrated as the sequence of a vitivirus detected in one Italian accession was almost identical with sequence obtained from a New Zealand accession of the same cultivar imported from China (Cohen and Blouin, personal communication). This demonstrated the risk of transfer of viruses in scionwood.

PCR and/or serological detection methods are available for all known *Actinidia* viruses and consequently it is possible and advisable to test for all known viruses when moving small amounts of material internationally or selecting mother stock for multiplication. To minimize virus spread within orchards or specific geographical locations it is desirable to instigate a high health scheme for seedlings and bud wood in order to produce certified propagation material. While visual assessment is likely to be an important part of this it is not sufficiently reliable to be used on its own. However, routine screening of all bud wood and seedlings for all known viruses is unlikely to be a practical proposition. Consequently there is a need to identify those viruses which pose the greatest risk both in terms of impact on individual trees and their ability to spread. Further information is required to determine whether specific sanitation methods are economically justified.

## Conclusions

In conclusion, *Actinidia* is a natural host to a wide range of viruses, but there is currently very little information on the biological properties (e.g. disease symptoms, host range, vectors) of most of the viruses isolated from *Actinidia* species; consequently it is not possible to predict the precise effects of the various viruses on different *Actinidia* species and cultivars (Pearson *et al*., 2011).

Compared to our knowledge of viruses of most traditional tree crops, our current knowledge of kiwifruit viruses is still limited and a number of assumptions have been made based on the known properties of virus isolates from other hosts. Even though the viruses detected so far may be only a small proportion of the viruses able to infect kiwifruit, they represent a sufficiently wide range of modes of transmission to show that preventing the spread of kiwifruit viruses is likely be both difficult and complex.

It is clear that nursery practices and exchange of plant material play a key role in virus dissemination at both local and international level, therefore sanitary selection and, eventually, sanitation are the only functional means for producing certified stocks. Additionally, virus control depends on preventive measures aimed at reducing vector populations and sources of inoculum. Following the same rational, breeding programs must be assisted by viral testing in order to exclude presence of pollen or seed-transmitted viruses from parental lines. Our knowledge of viruses infecting kiwifruit is currently insufficient to provide the needed support to all of the preventive measurements mentioned above. International research programs aimed at discovering new viruses in *Actinidia* spp. and studying their biological and molecular proprieties within different countries are therefore necessary to fill this gap.

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# Appendix A:

# Invited Review
# Viruses of kiwifruit (*Actinidia* species)

**INVITED REVIEW**

# VIRUSES OF KIWIFRUIT (*ACTINIDIA* SPECIES)

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## SUMMARY

Kiwifruit (*Actinidia deliciosa*) was introduced to New Zealand more than one hundred years ago and the New Zealand-raised cv. Hayward is now the dominant cultivar grown worldwide. Further accessions of kiwifruit seed and scionwood have been sourced from China for research and breeding. In one importation consignment, the first virus naturally infecting kiwifruit, *Apple stem grooving virus* (ASGV), was identified following symptoms observed in quarantined plants (2003). Since that time a further 12 viruses have been identified in kiwifruit. We classify these 13 viruses into three groups.

The first group comprises the non-specialist viruses and includes *Alfalfa mosaic virus* (AMV) and *Cucumber mosaic virus* (CMV) both members of the family *Bromoviridae*. The group also includes a further five viruses that appear to have limited effect on kiwifruit: two tobamoviruses, *Ribgrass mosaic virus* (RMV) and *Turnip vein clearing virus* (TVCV); a tombusvirus, *Cucumber necrosis virus* (CNV); a novel potexvirus; and *Apple stem gooving virus* (ASGV, genus *Capillovirus*). Most of the viruses classified in this first group are cosmopolitan and sometimes orchard weeds provide reservoirs for infection.

The second group comprises the kiwifruit-adapted viruses. This group includes three novel viruses. i.e. two vitiviruses, *Actinidia virus A* (AcVA) and *Actinidia virus B* (AcVB), and a citrivirus closely related to *Citrus leaf blotch virus* (CLBV). In addition, preliminary evidence of a novel virus belonging to the *Closteroviridae* family has been obtained.

The third group of viruses induces disease in kiwifruit. To date only two viruses have caused significant damage to kiwifruit within commercial orchards. In New Zealand, *Cherry leaf roll virus* (CLRV) has been detected on kiwifruit associated with symptoms including leaf spots, fruit malformation, reduction in yield, bark cracking and cane wilting. *Pelargonium zonate spot virus* (PZSV) has been detected in Italy associated with severe symptoms on leaves and fruit.

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## INTRODUCTION

In 1904, Isabel Fraser introduced the first kiwifruit seed to New Zealand, and by 1910 the plants raised by a friend, Alexander Allison, produced the first fruit outside China (Ferguson and Bollard, 1990). *Actinidia deliciosa* cv. Hayward was selected around 1925 and kiwifruit production started in New Zealand by 1930 (Ferguson and Bollard, 1990). The original name 'Chinese gooseberry' was replaced by 'kiwifruit' when the first fruit were exported to the USA in 1959 (Ferguson and Bollard, 1990). The name 'kiwifruit' is now often used for all species within the genus *Actinidia*. Until 2000 *A. deliciosa* 'Hayward' was the cultivar of choice, and almost all the international trade in kiwifruit was of this one cultivar. When facing overproduction in the early 1990s, the New Zealand industry innovated and assessed the commercial potential of another species, *Actinidia chinensis* (Ferguson and Huang, 2007).

*A. deliciosa* has fruit with green flesh and hairy skin, while *A. chinensis* has smooth-skinned fruit and, usually, yellow flesh. Other differences include fruit flavour, flower size, shoot hairiness, geographic distribution, chromosome number, and leaf shape (Ferguson and Bollard, 1990). The introduction of the yellow-fleshed *A. chinensis* cv. Hort16A, marketed under the Zespri Gold Kiwifruit brand, changed the industry by offering a product that complemented, rather than competed with, cv. Hayward resulting in increased consumption (Anonymous, 2012). Since 2000, most newly planted orchards in New Zealand have been *A. chinensis* and cv. Hort16A now represents about 26% of the New Zealand export of kiwifruit (Anonymous, 2012). Other yellow-fleshed *A. chinensis* have now been commercialised in a number of countries, including cvs Jintao or ENZAGold™. About 30% of kiwifruit planted in China is now *A. chinensis* (Ferguson and Seal, 2008). The success of the yellow and, subsequently, a red-fleshed *A. chinensis*, combined with the need to intro-

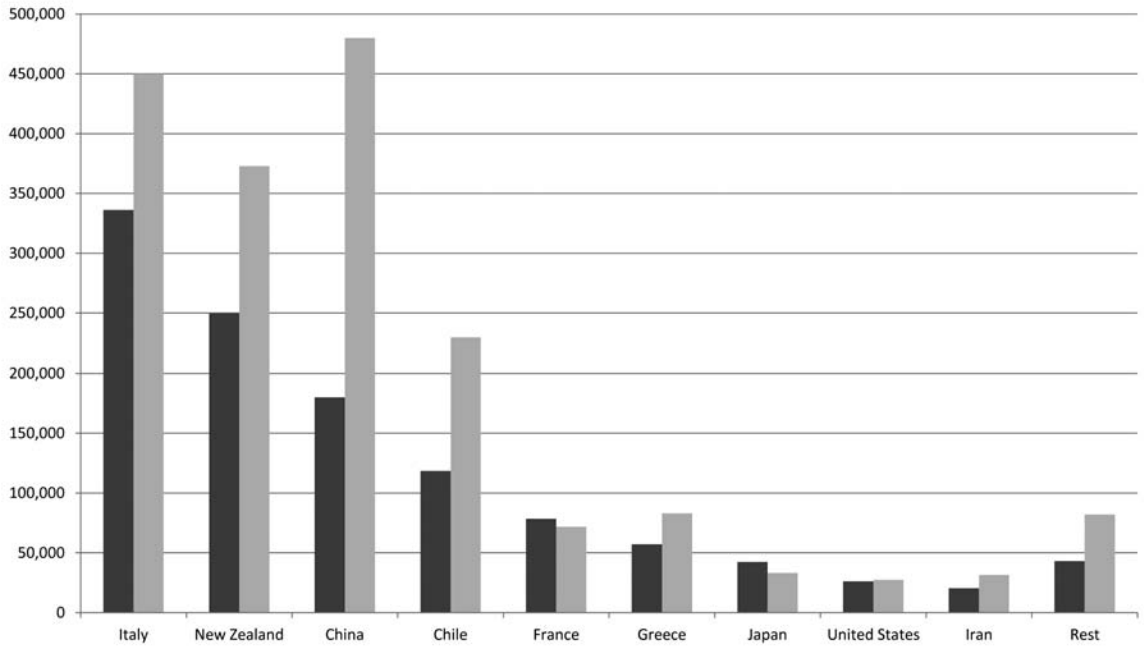


**Fig. 1.** Kiwifruit production worldwide past 20 years (after Belrose Inc., 2012). Data from world kiwifruit review 2012 based on predicted production for 2012. Production for 1999-2002 is in dark grey with a total production of 1,152,578 tons. The predicted production for 2009-2012 is in light grey with a total of 1,862,487 tons.

duce novelty into the market, has intensified breeding programmes in New Zealand, Italy and China. This breeding activity has resulted in more plant movement between countries. Despite the development of new varieties, 'Hayward' is still the predominant fruit traded internationally, comprising an estimated 90–95% of the worldwide kiwifruit market (Ferguson and Seal, 2008).

International kiwifruit production is concentrated in relatively few countries. The top four countries are China, Italy, New Zealand and Chile, which collectively produce more than 80% of the world's kiwifruit crop; the top ten producing countries represent more than 96% of the world supply (Anonymous, 2012) (Fig. 1). Commercial production of kiwifruit in China has increased steadily over the past two decades; now, China is the biggest producer, with more than 25% of the world's production. This has contributed to the 62% increase in total world production over the past 10 years (Anonymous, 2012).

Disease pressure is a new concept for the kiwifruit industry. Some fungal diseases were reported previously, such as *Armillaria novae-zelandii* in New Zealand (Horner, 1992); *Phomopsis* sp. in Greece (Elena, 2009); *Cadophora melinii* in Italy (Prodi *et al*., 2008); and verticillium wilt of gold kiwifruit in Chile (Auger *et al*., 2009). To date, these pathogens tend to be localised.

The recent detection of a virulent strain of *Pseudomonas syringae* pv. *actinidiae* in Italy and New Zealand (Ferrante and Scortichini, 2010; Everett *et al*., 2011) has had disastrous consequences for the production of *A. chinensis* cv. Hort16A. Some anticipate that all cv. Hort16A plantings in New Zealand, and possibly in other producing countries, could be removed because of the cultivar's vulnerability to the disease (Anonymous, 2012; Young, 2012) resulting in an urgent need for new resistant cultivars.

Although there were some reports of virus-like symptoms, no viruses were identified in kiwifruit before 2003. The first indication of a kiwifruit-infecting virus comes from New Zealand quarantine records in 1983. Gary Wood, from the then Department of Industrial and Scientific Research (DSIR, New Zealand), documented local lesions observed on *Chenopodium quinoa* after sap inoculation of kiwifruit imported from China and held in quarantine. The infected kiwifruit plants were either destroyed or died during thermotherapy (G. Wood, personal communication).

In the 1980s, as Italy was becoming an important kiwifruit producer with the second greatest area planted worldwide, there were no records of viruses infecting the crop. Caciagli and Lovisolo (1987) surveyed commercial orchards for potential viral diseases and collected samples from 100 symptomless *A. deliciosa* and one plant of *A. deliciosa* that showed chlorotic mottling. The extracts from these plants were mechanically inoculated into four herbaceous indicators (*C. quinoa*, *C. amaranticolor*, *Nicotiana glutinosa* and *N. clevelandii*). None of the 404 inoculated indicator plants displayed symptoms. Additionally, the authors challenged young *A. deliciosa* plants with 17 common viruses from Italy, including *Alfalfa mosaic virus* (AMV) and *Cucumber mosaic virus* (CMV). Only three viruses, *Tobacco necrosis virus*

(TNV), *Tobacco rattle virus* (TRV) and CMV, induced symptoms on the inoculated leaves of the kiwifruit, and only CMV moved systemically. The authors concluded that kiwifruit may be resistant to virus infections.

A few years later, during a survey in the Fujian Province in China, Lin and Gao (1995) identified one plant showing a "mosaic disease" attributed to an unidentified virus. Nitta and Ogasawara (1997) reported evidence of a graft-transmissible agent causing virus-like symptoms. Using cuttings from *Actinidia polygama* plants collected in the mountains of Hiroshima Prefecture (Japan) as rootstocks, they observed chlorotic spots and rings on the eight different *A. deliciosa* varieties used as male scions. In neither case was the causal agent identified.

In 2003, *Apple stem grooving virus* (ASGV) was identified in a kiwifruit import from China held in New Zealand quarantine (Clover *et al.*, 2003). This first virus identified in kiwifruit was detected by leaf symptoms, transmission electron microscopy (TEM) and mechanical transmission to herbaceous indicators, and identified by DAS-ELISA, RT-PCR and sequencing of amplicons. Other kiwifruit from the same consignment were subsequently studied further and new viruses were identified.

To date, the viruses discovered in kiwifruit can be divided in three groups. The first group comprises AMV, ASGV, CMV, *Cucumber necrosis virus* (CNV), *Ribgrass mosaic virus* (RMV), *Turnip vein clearing virus* (TVCV), and a novel potexvirus, tentatively named Actinidia virus X (AVX). These viruses are mostly ubiquitous/ cosmopolitan and, so far, do not show a detrimental effect on commercial kiwifruit. Most of these viruses are distributed worldwide over a large host range and have been detected in alternative hosts neighbouring kiwifruit orchards.

The second group comprises the putatively kiwifruit-specific viruses that, to date, are only known to have

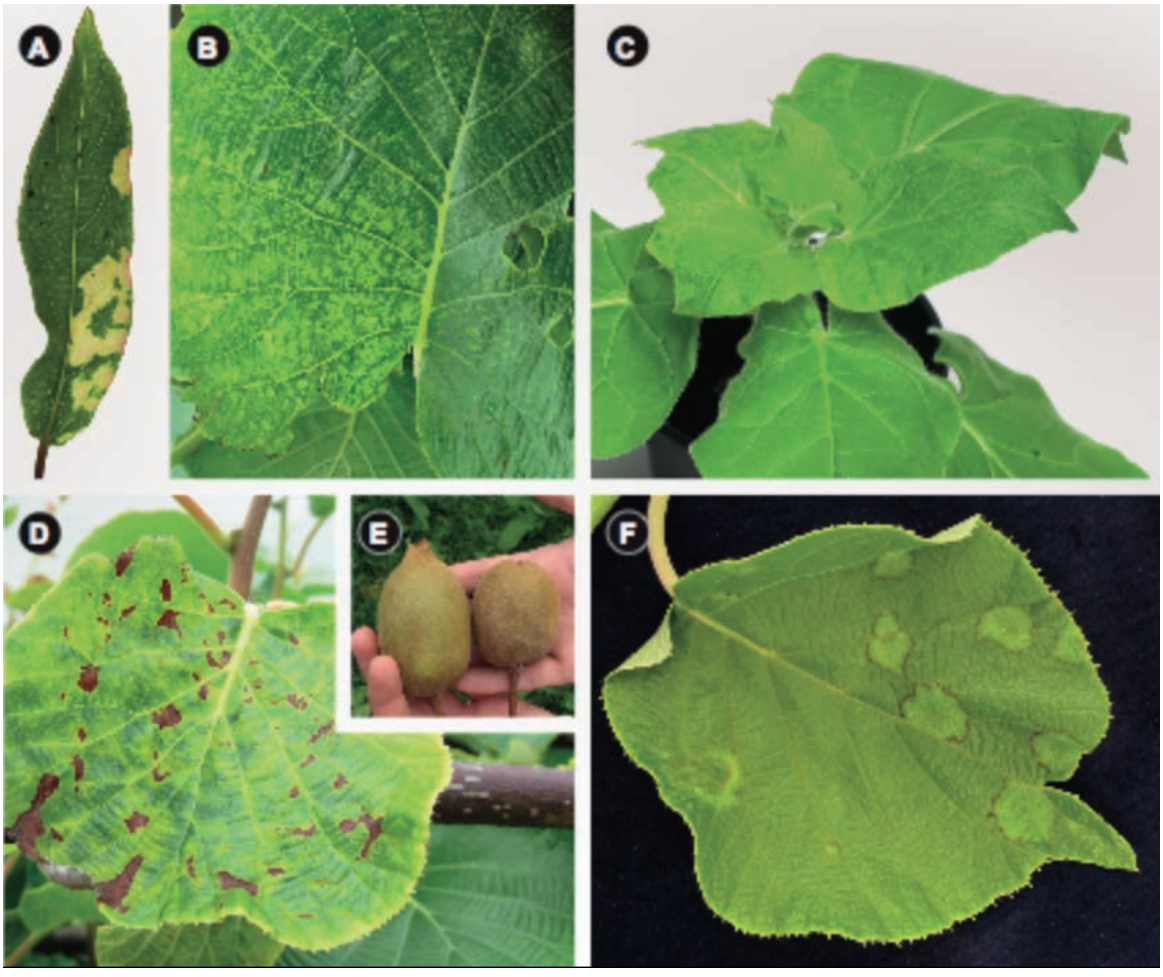


**Fig. 2.** A. Symptomatic leaf of *Actinidia glaucophyla* infected with *Alfalfa mosaic virus*. B. Symptomatic leaf of *Actinidia chinensis* infected with *Actinidia virus A*, *Actinidia virus B* and Actinidia citrivirus. C. Symptomatic *Nicotiana glutinosa* infected with Actinidia citrivirus. D. Symptoms associated with *Cherry leaf roll virus* in *Actinidia chinensis* cv. Hort16A. Chlorosis developing into necrosis on a leaf. E. Symptoms associated with *Cherry leaf roll virus* (CLRV) in *Actinidia chinensis* cv. Hort16A, a regular fruit on the left with a beak at the calyx end characteristic of cv. Hort16A, and fruit infected with CLRV on the right not showing the beak. F. Symptoms observed on leaves of *Actinidia chinensis* cv. Hort16A infected with *Pelargonium zonate spot virus*..

this single host or are likely to have a very limited host range. In this group we have identified two vitiviruses, *Actinidia virus A* (AcVA) and *Actinidia virus B* (AcVB), and a citrivirus closely related to *Citrus leaf blotch virus* (CLBV). There is also evidence of a novel virus from the family *Closteroviridae*, although the data for this virus are still being collected.

The third and most concerning group includes two viruses that have very recently been detected in kiwifruit. *Cherry leaf roll virus* (CLRV) in New Zealand and *Pelargonium zonate spot virus* (PZSV) in Italy both cause severe damage to the commercial crop. Almost 10 years since the first publication of kiwifruit virology, we describe now the 13 viruses detected in kiwifruit to

**Table 1.** Viruses infecting *Actinidia* species in nature: taxonomic allocation, epidemiology, geographical distribution and reference to kiwifruit.

| Virus name, (abbreviation), Genus, Family | Particle size | Symptoms on Kiwifruit, vectors | Host range | Geographical distribution in kiwifruit (other hosts) | First report in kiwifruit |
|---|---|---|---|---|---|
| Actinidia citrivirus, *Citrivirus, Betaflexiviridae* | Flexuous, 750–800 nm | Associated with vein clearing and mild mottling on leaves and interveinal chlorosis. No known vector. | Kiwifruit | China, New Zealand | Pearson *et al.* (2011) |
| *Actinidia virus A* (AcVA) and *Actinidia virus B* (AcVB), *Vitivirus*, *Betaflexiviridae* | Flexuous, 750–800 nm | Associated with leaf clearing, ringspots but can be latent. No known vector. | Kiwifruit | China, Italy, New Zealand | Blouin *et al*. (2012) |
| Actinidia virus X (AVX), *Potexvirus, Alfaflexiviridae* | Flexuous, 470-580 nm | Can be latent. Vector unknown. | Unknown | New Zealand | Pearson *et al.* (2011) |
| *Apple stem grooving virus,* (ASGV), *Capillovirus, Betaflexiviridae* | 620-700x12 nm *Actinidia* isolate = 680 nm | Interveinal mottling, chlorotic mosaics and ring-spots. No known vector. | Apple, pear, cherry, citrus and kiwifruit and nine dicotyledonous families | China, New Zealand (worldwide) | Clover *et al*. (2003) |
| *Alfalfa mosaic virus* (AMV) *Alfamovirus Bromoviridae* | Baciliform of different length (56, 43, 35 and 30 nm) and constant diameter of 18 nm | Mild symptoms on *A. chinensis.* Transmitted by aphids, seeds and pollen. | Very wide host range | New Zealand (worldwide) | Pearson *et al.* (2011) |
| *Cherry leaf roll virus* (CLRV) *Nepovirus Secoviridae* | Isometric particles *ca.* 28 nm in diameter | Necrotic spot on leaf, bark craking and cane wilting in severe infection. Cv. Zespri Gold Kiwifruit shape altered. Transmission through seed, pollen, grafting and mechanical inoculation. No known vector. | Wide host range | New Zealand (worldwide) | Woo *et al*. (2012b) |
| *Cucumber mosaic virus,* (CMV) *Cucumovirus, Bromoviridae* | Isometric *ca.* 28 nm in diameter | Chlorosis on *A. chinensis.* Transmitted by aphids, seeds and pollen. | Extremely wide | Italy, New Zealand (worldwide) | Pearson *et al.* (2009) |
| *Cucumber necrosis virus*, (CNV) *Tombusvirus, Tombusviridae* | Isometric, 31 nm in diameter | Symptomless in kiwifruit. Vectored by fungus *Olpidium radicale*. Not seed transmitted. | Cucumber, lettuce, tomato and kiwifruit | China, Italy, New Zealand (Canada, the USA, China, Italy, New Zealand) | Lebas *et al.* (unpublished) |
| *Pelargonium zonate spot virus* (PZSV), *Anulavirus, Bromoviridae* | Quasi-spherical, non-enveloped and diameter ranging from 25 to 35 nm | Concentric chlorotic/necrotic rings and line patterns. Transmitted by seed and pollen. | Large host range including pelargonium, tomato, pepper, artichoke, and kiwifruit | Italy (Australia, France, Italy, Israel, Spain, and the USA) | Biccheri *et al.* (2012) |
| *Ribgrass mosaic virus,* (RMV), *Turnip vein clearing virus* (TVCV), *Tobamovirus, Virgaviridae* | 300 x 18 nm rigid rod | Can be symptomless in kiwifruit. No known vectors, mechanically transmissible, possibly transmitted on seed. | Wide host range | China, New Zealand (worldwide) | Chavan *et al.* (2009) |

**Table 2.** Diagnostic tools: reagents for ELISA when available, and primers used and conditions for PCR assays.

| Virus name | ELISA | PCR | | | | |
|---|---|---|---|---|---|---|
| | | Forward primer | Reverse primer | Annealing (°C) | Amplicon size (bp) | Reference |
| Actinidia citrivirus | Dweet mottle antiserum Antiserum USDA253, (courtesy Dr. Richard Lee) | CLBV 1F AGCCATAGTTGAACCATTCCTC | CLBV 5R GCAGATCATTCACCACATGC | 58 | 425 | Chavan *et al.* (unpublished) |
| AcVA | Not available | AcVA 1F ATGATGGGGTGTTCTATGGGTGG CT | AcV 1R CTCATTCTCCAMCCRCARAAGAG | 55 | 269 | Blouin *et al.* (2012) |
| AcVB | Not available | AcVB 1F AATTCGGACCACTCCTGAGGC | AcV 1R CTCATTCTCCAMCCRCARAAGAG | 55 | 529 | Blouin *et al.* (2012) |
| AMV | Bioreba (Switzerland) Cat 140512-140522 Only reliable for symptomatic *Actinidia* tissue and herbaceous indicators | AMV for TGTCTCACTGATGACGTG | AMV rev CATACCTTGACCTTAATCCAC | 55 | 415 | Blouin *et al.* (2010) |
| ASGV | Bioreba Cat 150912 and 150922 | CTLV-AP CCTGAATTGAAAACCTTTGCTGCC ACTT | CTLV-AM TAGAAAAACCACACTAACCCGG AAATGC | 60 | 456 | Ito *et al.* (2002) |
| AVX | Rabbit polyclonal antiserum saised against purified virus (Plant and Food Research) | AVX-F (3963) AAGTCCGCAACACCTACCTG | AVX-R (4118) GGACAGACGATAGCAGCCTT | 58 | 175 | D. Cohen and A.G. Blouin (unpublished) |
| CLRV | Bioreba, Cat 150822 and 150812 | CLRV-F TGGCGACCGTGTAACGGCA | CLRV-R GTCGGAAAGATTACGTAAAAGG | 55 | 416 | Werner *et al.* (1997) |
| CMV | Bioreba, Cat 160612 and 160622 | CMV-F CTTTCTCATGGATGCTTCTC | CMV-R GCCGTAAGCTGGATGGAC | 54 | 885 | Felix and Clara (2008) |
| | | CMV nF (nested if required) ACTATTAACCACCCAACCT | CMV nR (nested if required) TTTGAATGCGCGAAACAAG | | Nested: 172 | |
| CNV | DSMZ (Germany), antisera AS-0130 | PCR1 Gral. Tombusvirus F1 AAGGGTAAGGATGGTGAGGA | PCR1 Gral. Tombusvirus R1 TTTGGTAGGTTGTGGAGTGC | PCR1 55 | PCR1 587 | PCR1 Harris *et al.* (2007) |
| | | CuNV-F791 (nested) CCTCGCAGAAGACCTTATGC | CuNV-R1002 (nested) GCCGACTCCTCCACTCCA | Nested-PCR 60 | Nested-PCR 215 | Nested-PCR Lebas *et al.* (unpublished) |
| PZSV | ADGEN Phytodiagnostics | PZSV2 F GATAAATTCAGAGCTCTCGG | PZSV2 R ATCTCTGCAGATTGTGTTCC | 55 | 997 | Ratti *et al.* (unpublished) |
| RMV and TVCV | Rabbit polyclonal antiserum raised against purified TMV (Auckland University) | AT2F AGACAGCAATTCTCAAACTTGT | AT 4R CGGTCGCATCATCAACAC | 55 | 223 | Chavan *et al.* (unpublished) |

date. This represents the first review of kiwifruit viruses, including images of symptoms (Fig. 2), a summary table of each virus (Table 1), and a summary of diagnostic tools including primer sequences and amplification conditions (Table 2).

## NON-SPECIALIST VIRUSES

***Alfalfa mosaic virus* and *Cucumber mosaic virus.*** AMV and CMV are two viruses infecting a very broad host range, with over 1200 plant host species in over 100 families for CMV (Douine *et al.,* 1979) and 300 species in 22 plant families for AMV (Hull, 1969). The addition of *Actinidia* species to their host range is not unexpected. Because of the damage CMV causes on some economically important crops, it was included in the "Top 10 plant viruses" in a recent molecular pathology review (Scholthof *et al.*, 2011). Both viruses belong to the family *Bromoviridae* and are efficiently vectored by a number of aphid species. They are also transmitted by seed and are easily transmissible mechanically. AMV is the type member of the genus *Alfamovirus* and has four bacilliform type particles (Fauquet *et al.*, 2005). CMV is the type member of the genus *Cucumovirus* and has icosahedral particles.

AMV was one of the first viruses detected and identified in kiwifruit in New Zealand (Pearson *et al.*, 2009). It was first detected in *Actinidia glaucophylla,* showing strong yellow mosaic patterns (Fig. 2A). Extracts from the chlorotic blotch easily transmitted the virus to a range of herbaceous indicator plants. In the same germplasm collection, AMV was also isolated from *Actinidia guilinensis* and *A. fortunatii* showing mottled and generally chlorotic leaves. In these hosts, the plants looked unthrifty and the virus symptoms were widespread in the block. The symptoms were observed in spring for four consecutive years. AMV and CMV have been found as a dual infection in both *A. glaucophylla* and *A. fortunatii,* and CMV was also detected in a single symptomless infection of *A. glaucophylla.*

AMV has only been detected once in *A. chinensis* in New Zealand. The plant showed a few leaves with very minor chlorosis and the symptoms could not be ob-

served the following year. Inoculation of AMV to *A. chinensis* seedlings induced foliar symptoms on one or two leaves above the inoculated leaf, but newer leaves were symptomless. CMV has been detected in Italy on one *A. chinensis* plant with pale mottling of the leaves.

AMV and CMV can be detected by RT-PCR in *Actinidia* sp. (Table 2). DAS-ELISA can also be used for both viruses but AMV can only be detected in symptomatic tissues. Both viruses are readily transmissible to a range of herbaceous indicators including *N. benthamiana*, *N. clevelandii*, *N. glutinosa*, and *N. occidentalis*.

These two viruses are similar in terms of their abundance in the surrounding weeds, and also by sharing the same vectors. Both are present worldwide and are likely to infect *Actinidia* sp. causing some concerns for the non-commercial species (*A. glaucophylla, A. guilinensis* and *A. fortunatii*). Fortunately, the viruses do not appear to have a detrimental effect on either *A. chinensis* or *A. deliciosa*. Their impact on these important crops is therefore negligible.

***Ribgrass mosaic virus* and *Turnip vein clearing virus.*** RMV and TVCV are two closely related species in subgroup 3 of the genus *Tobamovirus*, family *Virgaviridae*. Both viruses have 300 nm rod-shaped particles with positive sense, single-stranded RNA (ssRNA) (Adams *et al*., 2009). RMV was first reported from *Plantago* (Holmes, 1941) and has been variously referred as Holmes ribgrass virus, *Tobacco mosaic virus*-ribgrass strain, Crucifer TMV, and TMV Wasabi (Gibbs, 1999). It has been reported from at least 67 different species belonging to 15 diverse dicotyledonous and monocotyledonous families (Chavan *et al*., 2012). Symptoms include systemic chlorotic mottling, ring-like markings, chlorotic streaks along the veins and twisting of the petioles in *Plantago* species, vein clearing in turnip (Lartey *et al*., 1993), necrotic mosaic in tobacco and internal browning of tomato fruit (Oshima and Harrison, 1975). Tobamoviruses have no known natural vectors but the particles are stable and readily mechanically transmitted. They can also be carried and transmitted from the surface of seeds (Gibbs, 1977).

RMV was first detected in *A. deliciosa* and *A. chinensis* held in post-entry quarantine in New Zealand (Chavan *et al*., 2009) and the complete sequences of the isolates from *A. chinensis* (GenBank accession No. GQ401366.1) and *A. deliciosa* (GQ401365.1) were subsequently published (Chavan *et al*., 2012). RMV and TVCV were first reported in New Zealand from *Plantago* spp. (Cohen *et al.,* 2012). Subsequent studies have identified both viruses in *A. chinensis* in New Zealand, and TVCV has been identified in samples of dried leaf material of *A. chinensis* from both China and Italy (Cohen *et al.,* unpublished information). Both viruses were amplified by the primers designed to detect RMV (Chavan *et al*., 2012) and can only be distinguished by sequencing of the amplicons.

Symptoms on *A. chinensis* include chlorosis of leaf veins and adjacent tissue during spring and chlorotic mottles, mosaics, and ringspots during summer. Symptoms on *A. deliciosa* include chlorotic mottling or mosaic during spring and ringspots during summer months (Chavan *et al*., 2009). Some of the symptoms resemble those previously described in *Actinidia* infected with ASGV (Clover *et al*., 2003) and subsequent investigation has established that most of the plants were co-infected with other viruses (Chavan *et al*., unpublished information). Symptoms on mechanically inoculated indicators include local chlorotic lesions in *C. amaranticolor* and *C. quinoa,* systemic mosaic and distortion in *N. benthamiana,* systemic necrotic ringspots and chlorotic vein banding and dark green blistering and distortion in *N. clevelandii*, local necrotic lesions and systemic mottle in *N. glutinosa* and *N. occidentalis,* and mild systemic mottle in *Phaseolus vulgaris* (Chavan *et al*., 2009), but some of these symptoms may be caused by co-infecting viruses.

For routine diagnosis, RMV and/or TVCV can be detected in *Actinidia* leaf samples by conventional RT-PCR (Table 2). ELISA, using a rabbit polyclonal antiserum raised against purified TMV (M. Pearson, The University of Auckland), detected *Actinidia* isolates of RMV in herbaceous indicators but failed to detect the virus in infected *A. chinensis* and *A. deliciosa* plants (Chavan *et al*., 2009). There are no known arthropod vectors of tobamoviruses but they can survive in sap for prolonged periods (Oshima and Harrison, 1975). Tobamoviruses are highly infectious and readily spread by contact between infected and healthy plants or via machinery and human handling (Gibbs, 1977). Consequently, similar treatments to those recommended to prevent the spread of TMV, such as seed sterilisation using hypochlorite, should be used to prevent virus on seed coats from infecting seedlings during nursery operations (Cohen *et al*., unpublished information). Overall, RMV and TVCV do not appear to cause significant damage to commercial kiwifruit orchards.

***Apple stem grooving virus.*** ASGV is the type member of the genus *Capillovirus*, family *Betaflexiviridae*. Its genome consists of a positive-sense ssRNA of 6,496 nucleotides (excluding the polyA-tail) enveloped in a flexuous, filamentous particle of 620-700x12 nm (Yoshikawa, 2000). Citrus tatter leaf virus (CTLV) is regarded as an isolate of ASGV, being indistinguishable from it biologically, serologically and in genome organization (Yoshikawa, 2000). The main crop hosts are apple, European pear, Japanese pear, Japanese apricot, citrus and lilies, and experimentally it infects more than 40 species in 17 plant families (Yoshikawa, 2000). It is probably found wherever apples are grown and natural spread has also been reported in citrus in China and

Japan (Yoshikawa, 2000). Some *Lilium* ASGV strains can infect *Citrus*, and a *Pyrus* isolate infects *Citrus* (Yoshikawa, 2000). The kiwifruit ASGV isolate from *A. chinensis* (AF522459) (Clover *et al*., 2003) has an identical genomic organization to strains from *Citrus*, *Malus* and *Lilium*, with a high degree of identity to *Citrus* (D16681), *Malus* (D14995) and *Lilium* (AB004063) isolates across the 32-terminal half (2,901 nt) of the genome. The coat protein and movement protein genes share a nucleotide identity of >95% with other strains of ASGV (Clover *et al*., 2003). The morphological, epidemiological, serological and molecular characteristics of the virus from *A. chinensis* are indistinguishable from those of ASGV from other hosts (Clover *et al.*, 2003).

ASGV in kiwifruit was first detected in *A. chinensis* budwood from Shaanxi province, (China), grafted onto healthy rootstocks of *A. chinensis* cv. Hort16A and grown in post-entry quarantine in New Zealand. The original source of the plants, within China, is not known. Infected plants developed interveinal mottling, chlorotic mosaics and ringspots (Clover *et al.,* 2003). However, these plants were subsequently found to be co-infected with RMV and vitiviruses (R.R. Chavan, unpublished information). ASGV is often latent in commercial *Malus* and *Citrus* although it can cause graft union necrosis, tree decline and death in some apple (Yanase, 1983) and citrus (Broadbent *et al*., 1994) rootstock/scion combinations. It is unknown whether ASGV results in significant yield losses in *A. chinensis* as it was detected in plants detained in post-entry quarantine under greenhouse conditions and observed for only a limited period of time (Clover *et al*., 2003). Some surveys for ASGV in *A. chinensis* have been carried out in New Zealand and ASGV was detected in extracts from some plants using RT-PCR and immunocapture-RT-PCR (ICRT-PCR). Sequencing of amplicons confirmed the presence of ASGV, but repeated extractions from the same plants gave variable results, indicating that the virus was unevenly distributed in the plants. Attempts to isolate ASGV from orchard plants by inoculation to herbaceous indicator plants have never been successful (Cohen *et al*., unpublished information).

ASGV is transmissible by grafting and mechanical inoculation to herbaceous plants. Vectors and natural means of field transmission are unknown for isolates from *Actinidia*, *Malus* or *Citrus* (Yoshikawa, 2000; Clover *et al*., 2003). ASGV is seed-transmitted in *Lilium longiflorum* and *C. quinoa* (Inouye *et al*., 1979) but it is unknown whether the *Actinidia* isolates are seed-transmissible. The *Actinidia* isolate was graft-transmitted to *A. deliciosa* and produced the same symptoms as in the original host. It was also mechanically transmissible to a number of herbaceous hosts (Clover *et al.*, 2003). The symptoms observed on *C. quinoa*, *Phaseolus vulgaris* and *Vigna unguiculata* are very similar to those described for isolates from other hosts (Inouye *et al*., 1979; Zhang *et al*., 1988; Yoshikawa, 2000).

For diagnostic purposes ASGV was successfully detected in infected indicator plants and directly from *Actinidia* samples by conventional RT-PCR using the primers (ML-F and ML-R, Table 2) of Ito *et al.* (2002). ASGV was also detected by ELISA, using ASGV antisera raised against apple strains of ASGV (Table 2), and ICRT-PCR. Both protocols were reliable but the ICRT-PCR was 50 times more sensitive than ELISA (Clover *et al*., 2003). Because the ASGV is thought to be transmitted in the field only by grafting, planting virus-free plants is the best means of controlling the virus. ASGV does not represent a threat to kiwifruit production.

***Cucumber necrosis virus.*** CNV (genus *Tombusvirus*, family *Tombusviridae*) is an isometric virus of 31 nm diameter containing ssRNA (Dias, 1972). CNV was first described in 1959 on cucumber plants from Canada which appeared stunted with severe foliar symptoms (McKeen, 1959). The virus is transmitted in soil by zoospores of the fungus *Olpidium radicale* [syn. *O. bornovanus*, *O. cucurbitacearum*; (Dias, 1970a, 1970b)] but not through seeds (McKeen, 1959). CNV can be mechanically transmitted to a wide host range including plants belonging to the families Amaranthaceae, Asteraceae, Chenopodiaceae, Cucurbitaceae, Fabaceae and Solanaceae (Dias, 1972). However, to date, the virus has only been found naturally to infect cucumbers (*Cucumis sativus*) in Canada (McKeen, 1959), lettuce (*Lactuca sativa*) and tomato (*Solanum lycopersicum*) in the USA (Obermeier *et al*., 2001), and kiwifruit (*Actinidia* spp.) in China, Italy and New Zealand (Lebas *et al*., unpublished information).

In 2009, *A. arguta* and *A. deliciosa* plants were bought from a commercial garden centre in Auckland (New Zealand) to be used as healthy controls for PCR. Both plants were found to be infected with CNV when tested by ICRT nested-PCR (Table 2). The 215 bp sequences obtained from both species were identical (KC478972, KC478973) and had 99% nucleotide identity with CNV isolates from Canada (M25270) and New Zealand (DQ663769). Subsequent testing of imported Chinese *A. deliciosa* (KC478971) and Italian *A. deliciosa* plants confirmed the presence of CNV in this material (B.S.M. Lebas, unpublished information). *Actinidia arguta* and *A. deliciosa* plants were propagated in a local nursery that provides plants to commercial garden centres all around New Zealand, so CNV is likely to be widely distributed within the country.

CNV causes necrotic spots, severe leaf distortion and stunting on greenhouse cucumber plants (McKeen, 1959). It elicits localized leaf necrosis on lettuce and was found in mixed infection with Lettuce necrotic stunt virus (LNSV, tenative species in the genus *Tombusvirus*) on tomato with leaf chlorosis and internal fruit necrosis in the USA (Obermeier *et al*., 2001). No

symptoms were observed on the two infected *Actinidia* plants from New Zealand or on the imported material from China and Italy. In addition, CNV was only detected by ICRT nested-PCR, suggesting it was present at a very low titre in all the *Actinidia* spp. plants tested. Therefore, it is likely that CNV is not a major pathogen of kiwifruit. Although CNV is detected in an increasing number of hosts, it has not been reported to cause any significant economic damage since the first report in 1959 (McKeen, 1959). CNV may have been present in New Zealand for some time. However, it has not been reported on any other crop species, although the vector *O. radicale* infects cucumber, tomato and beans (Pennycook, 1989). The impact of CNV on the kiwifruit production is unknown but is likely to be negligible.

**Actinidia virus X.** AVX is a novel putative potexvirus isolated on herbaceous indicator plants from three *A. chinensis* plants. The virus has flexuous particles of about 485 nm long and 12-13 nm width. Its sequence (KC568202) shows the typical organisation of a potexvirus with five ORFs. ORF1 (nt 26-4825) encodes the putative replicase of 1,599 aa with a calculated mass of 180 kDa. It contains the methyltransferase domain at the N-terminal, the NTPase/helicase domain in the central region and the RNA-dependant RNA-polymerase domain in the C-terminal region (Martelli *et al.*, 2007). ORF1 is followed by a short intergenic region of 52 nt and the triple gene block (TGB) formed by three overlapping ORFs; ORF2 (nt 4878-5585), ORF3 (nt 5554-5916) and ORF4 (nt 5753-6022) have a calculated mass of 26, 13 and 10 kDa respectively. ORF5 (nt 6041-6784) codes for a 26 kDa coat protein. Phylogenetic analysis showed the virus clustered with a subgroup comprising *Narcissus mosaic virus* (NMV), *Asparagus virus-3* (AV-3), *Malva mosaic virus X* (MaMV) and Scallion virus X (ScVX). The nucleotide identity on the full genome varied between 64 and 65% with these viruses, and between 57 and 59% nt identity with *Alstroemeria virus X* (AlsVX), *Lettuce virus X* (LVX) and *Pepino mosaic virus* (PepMV). AVX was easily mechanically transmissible to *N. benthamiana*, *N. clevelandii*, and *N. occidentalis,* and it induced systemic symptoms in *C. quinoa*.

Two out of the three isolations of the virus were made from samples of symptomatic kiwifruit. In these two plants, a vitivirus was also detected. The two symptomatic plants were destroyed after sample collection and resampling was not possible. The third detection was from a symptomless plant but re-isolation, RT-PCR and ELISA failed to re-detect the virus. It is possible that the virus is cryptic in kiwifruit in the same way that AlsVX is latent in *Alstroemeria* (Fuji *et al.*, 2005). Kiwifruit may not be the preferred host of AVX. The virus is probably distributed unevenly in kiwifruit plants and may occur at low titre, as it was only isolated on three occasions out of many hundreds of inoculations over the past 7 years. After purification of AVX from *N. occidentalis*, an antiserum was prepared from rabbit. Its successful use in indirect ELISA (plate-trapped antigen ELISA) was demonstrated from infected herbaceous indicators and leaves of *A. chinensis* seedlings that had been inoculated with the virus. AVX was detected at high titre in inoculated leaves of *A. chinensis* seedlings, but its titre gradually declined in new leaves over several months (Pearson *et al.*, 2011). Inoculated leaves on these seedlings showed veinal necrosis but no symptoms were observed on systemically infected leaves (D. Cohen and A.G. Blouin, unpublished information). AVX can also be detected by RT-PCR (Table 2). This virus has so far only been isolated from *Actinidia* spp on to *Nicotiana* spp and *C. quinoa* no further information is available on its host range and distribution. However, based on the absence of symptoms in systemically infected *A. chinensis* seedlings and the low incidence of detection, the impact of AVX is likely to be very low.

## KIWIFRUIT-ADAPTED VIRUSES

**Actinidia citrivirus**. The Actinidia citrivirus has a monopartite, linear, positive-sense, ssRNA genome of 8782 nt (JN900477) and shares 74% nucleotide identity with CLBV (AJ318061). The genome organisation is identical to that of CLBV, with three non-overlapping open reading frames and a 3' terminus poly(A) tract. ORF1 (nt 72-6035), the putative replicase polyprotein, includes methyltransferase, AlkB, OTu-like peptidase, papainlike protease, RNA helicase, and RNA-dependent RNA polymerase domains, typical of a citrivirus (Martelli *et al.*, 2007). It codes for 1987 aa and has a calculated mass of 230 kDa. ORF2 (nt 6035-7123) codes for a putative movement protein of 362 aa has a calculated mass of 40 kDa. An intergenic region of 55 nts follows ORF2 before the start codon of ORF3 (nt 7124-7178). ORF3 codes for a 40 kDa coat protein (358 aa). The 5' and 3' UTRs are 71 and 526 nt long, respectively (Chavan *et al.*, 2013). CLBV is the type and currently the only recognised member of the genus *Citrivirus*.

The Actinidia citrivirus has been detected only in kiwifruit scionwood material imported from China (Chavan *et al.*, 2013). In *A. chinensis* the virus is associated with a range of symptoms, including vein clearing and mild mottling on leaves and interveinal chlorosis during summer, although some infected accessions remained symptomless. All of the symptomatic kiwifruit plants infected with the Actinidia citrivirus were found to be co-infected, making if difficult to attribute the symptoms to one virus alone (Fig. 2B shows leaf symptoms of a plant co-infected with Actinidia citrivirus, AcVA and AcVB).

No attempt has been made to inoculate the Actinidia isolate to citrus, the only known natural host of CLBV.

The Actinidia citrivirus is transmitted by grafting in *Actinidia*, similarly to CLBV (Vives *et al*., 2001). CLBV is also transmitted by contaminated knife blades (Roistacher *et al*., 1980) and at a low percentage through seeds (Guerri *et al*., 2004), but so far there is no evidence that the Actinidia citrivirus can be mechanically transmitted by orchard operations and no seed transmission was observed within more than 300 *Actinidia* seedlings of an infected *A. chinensis* female parent; suggesting that if there is any seed transmission in kiwifruit, it would be at very low rate (D. Cohen and A.G. Blouin, unpublished information). The Actinidia citrivirus and CLBV have both been mechanically transmitted to a range of common herbaceous indicator plants including *N. benthamiana*, *N. clevelandii, N. glutinosa* and *N. occidentalis;* the citrus isolate of CLBV gave symptomless infections (Vives *et al*., 2008; Guardo *et al*., 2009) whereas the *Actinidia* isolate produced distinctive symptoms on *N. glutinosa* (Fig. 2C) (Chavan *et al.*, 2013).

Although Actinidia citrivirus isolates can be detected by ELISA using an antiserum against Dweet mottle virus [= CLBV (Antiserum USDA253, courtesy of Dr. Richard Lee] (D. Cohen and A.G. Blouin, unpublished information), and by PCR using primers designed from the coat protein gene of CLBV (Table 2), the Actinidia citrivirus shows several distinct differences. First, the symptoms induced in *N. glutinosa* (Fig. 2C). Second, all sequences of CLBV deposited in GenBank show very high similarity with one another, whereas the *Actinidia* citrivus isolates show considerable sequence variation. Third, phylogenetic analysis has shown that from the 3' end of ORF1 to the 3' untranslated region (UTR) (including all of ORF2 and ORF3) the citrus CLBV and the Actinidia citrivirus share 78% identity at the nt level and > 90% identity at the aa level. However, the 5' and 3' UTRs, as well as the 5' end of ORF1, show divergence of about 30% at the nt level (Chavan *et al.,* 2013). Based on current International Committee on Taxonomy of Virus (ICTV) demarcation criteria for sequence similarity within the family *Betaflexiviridae*, i.e. less than 72% nt identity or 80% aa identity in the CP or the polymerase gene (Adams *et al.,* 2011), Actinidia citrivirus is borderline for classification as a new species.

Since means of natural spread of the Actinidia citrivirus are unknown, control relies on the use of virus-free scionwood and rootstocks in combination with good hygiene to prevent the possibility of mechanical transmission via pruning. The impact of the virus is likely to be very low, mostly due to the lack of a vector.

***Actinidia virus A* and *Actinidia virus B.*** The genus *Vitivirus* was named after *Vitis* sp., host of the reference species *Grapevine virus A* (GVA). *Vitis vitifera* also hosts four additional vitiviruses, i.e. *Grapevine virus B*, *Grapevine virus D*, *Grapevine virus E* and Grapevine virus F (Adams *et al*., 2011; Al Rwahnih *et al.,* 2012). Most vitiviruses naturally infect a single host; the other natural vitivirus hosts currently known are mint (*Mint virus 2,* MV2) and heracleum (*Heracleum latent virus,* HLV) (Adams *et al*., 2011).

Two novel vitiviruses *Actinidia virus A* (AcVA) and *Actinidia virus B* (AcVB) were detected in kiwifruit by RT-PCR (Blouin *et al*., 2012). Both viruses have a monopartite, linear, positive-sense, ssRNA genome. AcVB genome was fully sequenced (JN427015) and is 7488 nt long and 7566 nt of AcVA were sequenced (JN427014) covering all the genome but the 5'UTR and the beginning of the ORF1. They share 64% nucleotide identity and each comprises five ORFs: ORF1 codes for the replication genes with a calculated mass of 195 kDa. Both sequences include conserved domain for a methyltransferase, an AlkB, a RNA helicase and a RNA-dependent RNA-polymerase in respective order from the amino terminus to the carboxyl terminus as described for the genus in Martelli *et al.* (2007); AcVA has a lysine-rich insert between motifs I and II of the methyltransferase that is not present in other vitiviruses, including AcVB. ORF2 codes for a putative protein of unknown function and has a calculated mass of 25 and 27 kDa for AcVA and AcVB respectively. This is the most divergent gene of the virus with only 16% aa identity between them and no homology to any protein from GenBank; ORF3 (nt 5704-6597 and 5698-6570) codes for a movement protein with a calculated mass of 33 and 32 kDa respectively, and share 56% aa similarity. ORF4 (nt 6515-7111 and 6488-7084) codes for the coat protein of a calculated mass of 21 kDa for both viruses. This is the most conserved gene of the viruses and AcVA and AcVB share 75% aa in common and are less than 70% aa similar to the closest vitiviruses (GVB and HLV). ORF5 (nt 7112-7429 and 7085-7405) codes for a putative RNA binding (RNA silencing inhibitor) protein of a calculated mass of 12 kDa (Blouin *et al*., 2012).

As a consequence of the historical movement of plant material, the grapevine-infecting vitiviruses have been reported in most grapevine-growing regions. Vitiviruses are not known to be seed-transmitted and AcVA and AcVB have only been detected in accessions that were imported to New Zealand as scions, or in scions that have been grafted on to an infected plant (Blouin *et al*., 2012). AcVA and AcVB have also been detected in two Chinese scionwood accessions growing in Italy (D. Cohen and A.G. Blouin, unpublished information).

Inoculation of sap from symptomatic vines of *A. chinensis* induced symptoms on *N. occidentalis*. The coat protein was partially purified from herbaceous indicator plants and a few peptides common to GVB were identified by tandem mass spectrometry (Blouin *et al*., 2010). A survey of more material showed symptoms ranged from large ringspots, vein chlorosis and mottle to symptomless plants, but some of the infected plants could host more viruses (Fig. 2B showing symptoms from a

mixed infection including AcVA, AcVB and the Actinidia citrivirus). AcVA and AcVB were transmitted by grafting to *A. deliciosa* but the infected plants remained mostly symptomless (Blouin *et al*., 2012).

Grapevine vitiviruses are spread by mealybugs and scale insects. Vitiviruses are often detected as coinfections with a member from the family *Closteroviridae*. In grapevine, *Grapevine leafroll associated virus 1* (GLRaV-1 genus *Ampelovirus*) has been reported to be co-transmitted with the GVA (Hommay *et al.*, 2008) . On some occasions, the vitivirus can be transmitted alone. A recent study using a donor plant with mixed infection of GVA and *Grapevine leafroll associated virus 3* (GLRaV-3) found that the majority of the receiving plants were infected with GLRaV-3 alone (24%) or both viruses (31%), while only 2% were infected with GVA alone and 43% were not infected (Blaisdell *et al*., 2012).

In kiwifruit, no movement of the *Actinidia* vitiviruses has been observed in New Zealand other than by grafting and all the positive vines could be linked to an import of scionwood from China (Blouin *et al.,* 2012). Some of the plants had been imported for several decades. This lack of movement suggests that the virus is present either without its helper virus or without efficient vectors. All the novel vitiviruses were detected in co-infection. It is possible that both viruses share a common vector (before the introduction to New Zealand), resulting in co-infection; however, it is expected that both viruses may also exist as single infections in the wild.

A virus that may potentially assist the natural transmission of Actinidia vitiviruses was identified by next generation sequencing (NGS) from a consignment of kiwifruit imported from China and held in quarantine in New Zealand. This virus has the characteristics of a member of the family *Closteroviridae*, but the sequence analysis shows that it is distant from any characterised species of this family (Blouin *et al*., unpublished information). Based on the heat shock protein 70 (HSP70), the most conserved gene within the family *Closteroviridae*, the closest relative (37% amino acid identity) was Olive leaf yellowing associated virus (OLYaV) (AJ440010), an unclassified member of this family. Further characterisation of this novel virus, including full sequence and transmission studies, may clarify its possible role as a helper virus.

The impact of vitiviruses on kiwifruit largely depends on their capacity to move and is therefore low in New Zealand. It is also too early to assess the impact of the novel putative closterovirus.

## DISEASE-INDUCING VIRUSES

***Cherry leaf roll virus.*** CLRV is an established species within subgroup C of genus *Nepovirus*, family *Secoviridae* (Sanfaçon *et al*., 2012). CLRV has been reported to be present in North America, Chile, Peru, Europe, China, Japan, Australia and New Zealand (Woo *et al*., 2012a). In addition to its worldwide distribution, the virus also has a wide natural and experimental host range, infecting members of more than 36 plant families (Walkey *et al*., 1973; Rebenstorf *et al*., 2006). This includes a variety of wild and cultivated, herbaceous and woody plant species. Unlike most nepoviruses, CLRV does not appear to be transmitted by soil-inhabiting nematodes. However, the virus has been documented to be transmitted by seed, pollen, grafting and mechanical inoculation to herbaceous hosts (Woo *et al*., 2012a).

CLRV has a bipartite genome of two positive-sense, ss-RNA molecules. Each RNA molecule is encapsidated separately in an isometric particle that is about 28 nm in diameter. Both RNA molecules are required for virus infection (Le Gall *et al*., 2005). RNA-1 and RNA-2 have structural organization typical of the genus and comprise 7905 and 6511 nt, respectively (Eastwell *et al*., 2012).

CLRV was first described in sweet cherry in England (Posnette and Cropley, 1955). Subsequently, it was found to cause leaf rolling and plant death in cherry (Cropley, 1961) and a range of other plant species including elderberry, olive, raspberry, rhubarb, walnut and a number of other shrub, tree, weed and ornamental species (Büttner *et al*., 2011; Woo *et al*., 2012a). CLRV was isolated from a *A. chinensis* cv. Hort16A orchard in which vines were showing necrotic symptoms on leaves (Fig. 2D), as well as cane die-back and bark cracking. Some of the fruit from the infected vines do not have the beak at the calyx end that is characteristic of the Hort16A cultivar (Fig. 2E). Additionally, the fruit from infected vines are uneven in size, and the crop yield is reduced. Extracts from symptomatic leaves inoculated to herbaceous indicators induced large necrotic lesions on *N. occidentalis* and ringspots on *N. tabacum*. The virus was identified by RT-PCR and sequencing. The sequences obtained from infected kiwifruit (JN371141) closely match those of an isolate from raspberry in New Zealand (Jones and Wood, 1978), and described as group C (Rebenstorf *et al*., 2006). Detection in symptomatic material is also possible with DAS-ELISA (Table 2). CLRV was also detected in *Rumex* spp. (JN371148) directly below the infected vines using DAS-ELISA. A mechanism for the movement of the virus between different hosts has not yet been identified. Within kiwifruit, the virus seems to spread along the row, suggesting a possible mechanical spread by pruning/girdling equipment. All these characteristics make CLRV a potential threat for kiwifruit production and future studies are required to understand fully its ecology.

***Pelargonium zonate spot virus.*** PZSV is the type species and the single member of the *Anulavirus* genus

within the *Bromoviridae* family (Bujarski *et al*., 2012). Amazon lily mild mottle virus, a new virus, isolated from an Amazon lily plant, has been recently described and proposed as new anulavirus species (Fuji *et al*., 2012). PZSV was described as *Tobacco streak virus* when first detected on tomato plants in southern Italy (Martelli and Cirulli, 1969) and later designated as PZSV when isolated from *Pelargonium zonale* (Quacquarelli and Gallitelli, 1979). This virus has been reported on tomato, pepper and weed species from Italy, Spain, France, the USA, Israel and Australia (Gallitelli, 1982; Luis-Arteaga and Cambra, 2000; Gebre-Selassie *et al*., 2002; Liu and Sears, 2007; Escriu *et al*., 2009; Lapidot *et al.,* 2010; Luo *et al*., 2010). As well as tomato, pepper and geranium, PZSV also naturally infects *Cynara cardunculus* var. *scolymus* (globe artichoke), *Capsella bursa-pastoris*, *Chrysanthemum segetum*, *Diplotaxis erucoides*, *Picris echioides, Sonchus oleraceus, Cakile maritima.* PZSV has been transmitted to herbaceous plants in 29 species, within nine dicotyledonous families, by mechanical inoculation (Martelli and Cirulli, 1969; Gallitelli *et al*., 1983).

Recently, PZSV has been detected in several symptomatic kiwifruit plants (*A. chinensis* cv. Hort16A) in Italy, from two orchards located in the Emilia-Romagna region. Infected plants showed chlorotic and necrotic rings on leaves (Fig. 2F) and depressed areas on the fruits that resulted in deformation of the berries (Biccheri *et al.,* 2012). Four infected plants were identified during 2011 and three additional plants were identified in 2012. Symptoms appeared early in the spring and remained evident until the end of the season in plants with severe infection but disappeared at the beginning of summer in plants with mild or sectorial infection. Moreover, cuttings obtained from symptomatic plants developed infected but symptomless leaves suggesting that a long incubation period, and therefore high viral titre, may be necessary for symptom expression.

Particles of PSZV are non-enveloped and quasi-spherical, with a diameter of 25-35 nm, and the coat protein is about 23 kDa (Gallitelli *et al*., 2005). The sequence of the complete genome has been obtained from the Italian tomato isolate; it is divided into three RNA species encoding four proteins (Finetti-Sialer and Gallitelli, 2003). RNA-1 is 3383 nt long, with a single ORF encoding a polypeptide which contains conserved motifs of type I methyltransferases and of the helicases of superfamily 1. RNA-2 is 2435 nt long and encodes a polypeptide (ORF2) showing identity to the RNA-dependent RNA polymerases of positive-strand RNA viruses. RNA-3 is 2659 nt long and contains two ORFs. The product of ORF3a shows similarities with the 30K superfamily of virus movement proteins and ORF3b encodes the viral coat protein, which is expressed via the subgenomic RNA-4 (Finetti-Sialer and Gallitelli, 2003; Gallitelli *et al*., 2005).

Poor data are available on the variability within PZSV isolates. High amino acid identity has been reported between Italian and Israeli tomato isolates (93% ORF 1a, 97% ORF 2a, 98% ORF 3a and 96% ORF 3b) (Lapidot *et al.*, 2010). Similar results have been obtained comparing the Italian isolates from tomato and kiwifruit (92, 99, 98 and 100% aa identity, respectively).

PZSV induces conspicuous concentric chrome-yellow bands in the leaves of *P. zonale*, from which its name is derived, and is the causal agent of a severe tomato disease characterized by concentric chlorotic/necrotic rings and line patterns of leaf stems and fruits together with plant stunting, leaf malformation, and reduced fruit set, which often result in plant death (Gallitelli, 1982). Data from preliminary studies on PZSV-infected kiwifruit plants suggest that the virus decreases vigour year by year and then productivity of the plants. Moreover, infected fruit exhibit progressive decreased metabolic activity and significant reduction of cell wall water content, indicating early senescence of tissues in PZSV-infected fruit compared with uninfected samples. The virus can be successfully transmitted from *A. chinensis* to indicator plants, including *C. quinoa*, *N. benthamiana*, *N. glutinosa* and *N. tabacum*, by mechanical inoculation during spring but efficiency decreases during summer or autumn.

PZSV can be detected directly from symptomatic kiwifruit tissues by ELISA, dot blot DNA hybridization and RT-PCR (Table 2), and in symptomless plants by RT-PCR.

PZSV is seed-borne in *Diplotaxis erucoides* and *N. glutinosa*. The virus is associated with the pollen and transmitted by thrips feeding on flowers of susceptible hosts (Vovlas *et al*., 1989; Gallitelli *et al*., 2005). In tomato, PZSV is transmitted by seed, with efficiency of 29%, and by pollen, although infected pollen cannot transmit the virus to mother plants, only to the seed (Lapidot *et al*., 2010).

No data are available about transmission from herbaceous host to kiwifruit and whether transmission occurs naturally between kiwifruit plants. New studies are therefore necessary to better investigate the biological and molecular proprieties of PZSV that infect kiwifruit and its role as a causal agent of disease in *Actinidia* sp. With regard to the symptoms in the commercial orchard, PZSV is an important pathogen to manage. Further study will assess its spread efficiency, which will determine the seriousness of the disease.

## CONCLUSION

Worldwide, the kiwifruit industry is relatively new and is based on mainly two species: *A. deliciosa*, representing the vast majority of the commercial production;

and *A. chinensis,* which comprises most of the newest cultivars. Cultivars of these two species are among the first from a recently domesticated plant family which holds promise for many further new commercial cultivars (Ferguson and Seal, 2008). Likewise, research on kiwifruit viruses is in its infancy. The viruses listed in this review were identified from only four laboratories, three in New Zealand and one in Italy, and all have been identified in kiwifruit over the past decade. The increased interest in disease-resistant cultivars of kiwifruit as well as the recent discovery of pathogenic viruses should stimulate further research. New technologies such as NGS will probably identify many new viruses, especially since this method does not require prior knowledge about infecting viruses. Such technologies will be useful to identify more RNA and DNA viruses and/or viroids, and to pinpoint rapidly the cause of disease when present, but could also uncover latent viruses, and potentially viruses that are beneficial (Roossinck, 2011). A major challenge is to undertake the basic research on the virus ecology so that vectors, host range and impacts on the plant host can be characterised.

We describe here 13 viruses that have been isolated from kiwifruit. Many of these viruses are not associated with important symptoms and/or spread (the non-specialists) and are not considered to be economically important in commercial orchards. Of the 13 viruses presented in this review, five (AVX, Actinidia citrivirus, AcVA, AcVB and a novel putative closterovirus), to date, have not been isolated from another host. With the exception of AVX, these are putatively kiwifruit specialists, as they are related to viruses that have a narrow host range. These kiwifruit-specialist viruses mostly cause leaf symptoms, but can also be latent. However, since these viruses have only been studied in non-commercial orchards, their effect on yield and plant longevity is unknown. No vector of these viruses has been identified yet and no movement to new kiwifruit plants has been observed except by grafting. Since it is likely that these viruses originate from wild kiwifruit populations in Asia, insect vectors are probably present in these countries. These specialist (or host adapted) viruses can infect plants without symptoms and would be easily overlooked and propagated within nurseries or orchards. The specialist viruses might pose a risk to kiwifruit growing within new environments if they infect new cultivars, interact with other viruses, or if a vector is present.

The third group of kiwifruit-infecting viruses at present comprises CLRV and PZSV, two viruses that pose more serious threats with respect to symptoms and spread. These two viruses are pollen-borne and seed-transmitted, although it is not yet known if this occurs in kiwifruit. Although the two viruses were identified recently, they have already been associated with significant symptoms, with consequences for yield. It is too early to assess the spread of these virus infections, but they are being monitored.

Kiwifruit virus research was initiated following the identification of ASGV during quarantine surveillance. Subsequent research identified the presence of Actinidia citrivirus, AcVA, AcVB, RMV and CNV in the same consignment of plant material (Chavan *et al*., unpublished information). The rigorous quarantine system in place in New Zealand has therefore demonstrated its importance. This review compiles a list of diagnostic tools that are now available to researchers and research laboratories as well as quarantine facilities for the current list of viruses known to infect kiwifruit. Table 2 indicates that RT-PCR is the most sensitive and reliable method for detection of these viruses in kiwifruit, although ELISA has also been widely used as a routine detection method.

The identification of these 13 viruses that can infect kiwifruit has important repercussions for orchard management, especially for nurseries that propagate kiwifruit. It is important to have nucleus stock plants that are free of known viruses. These health precautions should preclude the chance of infection from the specialist group of viruses. However, for PZSV and CLRV there is a potential for transmission from reservoir hosts to kiwifruit and subsequent spread by pollen or mechanical transmission that needs to be better investigated. Infected plants should be removed and equipment should be cleaned after use on infected vines. These virus infections are of sufficient importance that infections should be confirmed by local diagnostic laboratories and/or reported to local phytosanitary agents to determine whether further actions are required.

Kiwifruit breeding has a remarkable depth of genetic variation to exploit for new commercial attributes such as flavours, colours and nutritional benefits (Ferguson and Huang, 2007). The germplasm should also be screened for those vines that are either resistant or tolerant to each of these 13 viruses. The range of symptoms observed to date for some individual viruses on different cultivars suggests that there is potential for such virus resistance or tolerance. To date the industry has been fortunate to have selected a cultivar, *A. deliciosa* cv. Hayward, that has shown very good resistance or tolerance to disease in general and viruses in particular. Currently there is a need for tolerance to the bacterium *P. syringae* pv. *actinidiae* but screening for virus tolerance would also be prudent. This review provides a starting point for further studies to screen, identify and research viruses and plant-virus interactions in kiwifruit.

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# Appendix B

# Materials and Methods

## A.1 Organisms

**Test plants**

Host plants generally used for these research studies are:

- ***Chenopodium quinoa*** **and** ***Chenopodium armanticolor***: belong to *Chenopodiaceae* family and are host of many viral species showing symptoms ranging from chlorotic, yellow or necrotic local lesions in leaves to systemic symptoms such as leaf deformation and mosaic.
- ***Nicotiana benthamiana, Nicotiana tabacum, Nicotiana glutinosa*** **and** ***Nicotiana Occidentalis :*** are a systemic host for many viral species and belong to the *Solanaceae* family.

**Bacteria**

Bacteria generally used are:

- ***Escherichia coli*** strain **MC1022:** it is used for cloning and amplification. This strain allows blue/white colony screening in presence of IPTG and Xgal.
- ***Agrobacterium tumefaciens*** strain C58C1: this bacterium is used for agroinfiltration and carries rifampicin selectable marker.

## A.2 RNA and DNA processing

**Isolation and electron microscopic identification of virus**

Virus particles from infected tissues were isolated following the method of Turina *et al.* (2007) with a few modifications. The frozen or fresh leaves from infected plants (100 g wet weight) were homogenized in a blender in two volumes of extraction buffer (0.25M K-Phosphate pH 6.4, 1% sodium metabisulfite and 1mM of EDTA). After filtration through Miracloth (CalbioChem), in the homogenate was added 1% of Triton and stirred at 4°C for 1h. The homogenate was centrifuged at 9,300 x g for 20 min, the supernatant was subjected to ultracentrifugation in a Beckman 35Ti rotor at 95,000 x g for 5 h (60ml for each tube) (Beckman Instruments Inc). Each resulting pellet was

dissolved in 1 ml of K-Phosphate buffer 0.25M and after a passage with homogenizer, layered onto a 2 ml of 20 % sucrose cushion prepared in the same buffer and a second centrifugation was carried out at 250,000 x g for 2h in a 60Ti rotor (Beckman Instruments Inc) (50 ml of each tube). The homogenates were collected in a single tube and the centrifuge at 250,000 x g in a 60Ti rotor (Beckman Instruments Inc) was repeated. The resulting pellet was dissolved in 300 µl of K-Phospate buffer 0.25M and purified again by centrifugation through 10%-50% sucrose gradient and centrifuged at 250,000 x g for 1h 30 min in a SW41Ti rotor (Beckman Instruments Inc). Bands in the bottom of tube were collected, diluited in 0.25 M Potassium Phospate buffer and centrifuged at 250,000 x g for 2h in a 60 Ti rotor (Beckam Instruments Inc). The resulting pellet was resuspended in 100 µl of Potassium Phospate buffer 0.25M. Purity of the viral suspension was cheked by transmission electron microscopy.

Molecular weight of the proteins of virus particle was determined by sodium dodecylsulfate-polyacrylamide gel (SDS-PAGE 12%) stained in Coomassie brilliant blue.

**Trizol©total RNA extraction (Life technologies)**

Trizol© total RNA extraction was performed according to manufacturer's protocol. Fresh or frozen leaves and roots (100-200 mg) were crushed in a sterile 1.5 ml eppendorf with 1 ml of Trizol buffer. After 5 min at room temperature, 200 µl of chloroform are added and tubes are vigorously shaken by hand and incubate again at room temperature for 2 min. After centrifugation for 15 min at 12,000 g and 4°C, the aqueous phase is transferred in a fresh tube and 0.5 ml of isopropanol is added. RNA precipitation requires incubation for 20 min at room temperature then the RNA is pelleted, through centrifugation at 12,000g for 30 min at 4°C and washed with 1 ml of 70% ethanol. RNA pellet is dried 5 min and then resuspended in sterile water. RNA quality and quantity are analyzed on agarose gel and by spectrophotometer.

**Spectrum$^{TM}$ Plant Total RNA kit (SIGMA)**

Spectrum$^{TM}$ Plant Total RNA extraction was performed according to manufacturer's protocol. Fresh or frozen leaves (90-110 mg) were grinded in a fine powder in liquid

nitrogen using mortar and pestle. Pipet 500 µl of the Lysis Solution / 2-ME mixture (for each 500 µl of Lysis Solution add 10 µl of 2-mercaptoethanol (2-ME) on the tissue powder and vortex immediately for at least 30 seconds. Incubate the sample at 56 °C for 3-5 min. Centrifuge the sample at maximum speed for 3 min to pellet cellular debris. Pipet the lysate supernatant into a Filtration column (blue retainer ring) seated in a 2 ml collection tube. Close the cap and centrifuge at maximum speed for 1 min and save the clarified flow-through lysate. Pipet 750 µl of Binding Solution into the clarified lysate and mix immediately. Pipet 700 µl of the mixture into a Binding Column (red retainer ring) seated in a 2 ml collection tube. Close the cap and centrifuge at maximum speed for 1 min to bind RNA. Discard the flow-through liquid. Pipet 500 µl of Wash Solution 1 into the column. Close the cap and centrifuge at maximum speed for 1 min. Discard the flow-through liquid. Pipet 500 µl of Wash Solution 2 into the column. Close the cap and centrifuge at maximum speed for 1 min. Discard the flow-through liquid and pipet another 500 µl of Wash Solution 2 into the column. Close the cap and centrifuge at maximum speed for 1 min. Discard the flow-through liquid and centrifuge the column at maximum speed for 1 min to dry. Transfer the column to a new 2-ml collection tube and pipet 100 µl of Eluition Solution directly onto the center of the filter inside the column. Close the cap and let the tube sit for 1 min. Centrifuge at maximum speed for 1 min to eluate. RNA quality and quantity can be determinate by agarose gel and spectrophotometer.

**Purification and analysis of dsRNA from plant tissue.**

The dsRNA extraction were performed according to Valverde protocol (1990). Fresh or frozen leaves (3.5 g) were grinded in a fine powder in liquid nitrogen using mortar and pestle. The powder was transfered in a tube and add 8 ml of 1X STE (0.1 M NaCL, 0.05 M tris, 0.001 M EDTA), 1 ml of 10% SDS, 0.5 ml of bentonite (from a 2% aqueous suspension) and 9 ml phenol. The homogenate was shaked for 30 min and centrifuged at 8,000 x g for 15 min. Withdraw 10 ml of the upper aqueous phase and place in a 50-ml centrifuge tube (volume was adjusted to 10 ml with 1X STE if was not available). 2.1

ml of 95 % ethanol was added to each tube containing 10 ml of sample and mix well. Two 1-g portions of cellulose per sample was weighted and placed in 50-ml tubes and subsequently 25 ml of 1X STE containing ethanol 16% v/v were added (for 1 L: 100ml of 10X STE, 174 ml of 95% ethanol, 726 ml of distilled water). Two columns was prepared, using for each the barrel of a 20-ml plastic syringe plugged with a disk of Miracloth paper or glass wool. The cellulose suspension was mixed well, poured into the columns, and allowed the STE to drain through. The sample was added to one column and let it drain completely and the liquid from the column was discarded. The column was flushed with 40 ml of 1X STE containing ethanol, 16% v/v and refilled until all the buffer is used. The column was drained completely and washed again with 2.5 ml of 1X STE. Subsequently the sample was collected adding 10 ml of 1X STE and 2.1 ml of 95 % ethanol was added and the steps were repeated using the second column. After the last flush 6 ml of 1X STE was added to collect the sample and 0.5 ml of 3M sodium acetate (pH 5.5) and 20 ml of 95 % ethanol was added to each sample and left at least 2 h at -20 °C to precipitate the dsRNA. Sample was centrifuged at 8,000 x g for 25 min. The ethanol was poured off and the tubes were placed upside down to drain for about 15 min. To resuspend the dsRNA 50 µl of nuclease free water was addes to each tube. RNA quality and quantity was determinated by 1-1.5% agarose gel or 6% polyacrylamide gels.

**Poly(A) Tailing**

Poliadenilation (Poly(A) tailing) to the 3’ end is a method that can provide priming sites for the synthesis of first strand cDNA, and consisted in a rapid and efficient addition of poly(A) tails to the 3’ end of any RNA.

Poly(A) tailing was performed in order to determine the 3’ end of a viral RNAs. The Poly(A) tailing were carried out with Poly (A) Polymerase tailing kit (Epicentre Technologies, Madison WI), following the manufacturer's protocol.

In a 20 µl total volume were mixed 10 µg of total RNAs, 1 µl of Poly(A)Polymerase (4 U/ µl), 2 µl of Poly(A)Polymerase 10X reaction buffer (0.5M Tris-HCl pH 8, 2.5M NaCl and

0.1M $MgCl_{2)}$, 2 µl of 10mM ATP (supplied by manufacturer) and 20 units of RNasin® (40u/µl) (Promega, Madison, WI), the solution was incubated for 30 min at 37 °C.
The reaction was stopped with phenol-chloroform extraction and the RNA was precipitated with ethanol and 3 mM of sodium acetate pH 6, and resuspended in 10 µl of nuclease-free water.
The RT was performed using an oligo(dT) reverse primer (Promega, Madison, WI), and the PCR was performed using primer developed to known sequences near 3' the ends.

**Tobacco Acid Pyrophpshatase treatment**

Tobacco Acid Pyrophpshatase (TAP) can cleaves the pyrophosphate bond of the 5'-terminal methylated guanine nucleotide "cap" to get a ligation between the 5'-monophosphorylated terminus and 3'-hydroxylated terminus. TAP were performed using the Tobacco Acid Pyrophpshatase enzyme (Epicentre Technologies, Madison WI) according to manufacturer's protocol. Briefly, in a 50 µl total reaction volume were add 15 µg of total RNAs (extracted with Spectrum™ Plant Total RNA from Sigma-Aldrich according to the manufacturer's instructions), 0.5 µl TAP (5U/µl), 5 µl 10X TAP buffer (500 mM sodium acetate pH 6, 10mM EDTA, 1% 2-mercaptoethanol and 0.1% Triton X-100) and nuclease free $H_2O$ up to 50 µl. The reaction was incubated at 37°C for 1h. After extraction with phenol-chloroform, the RNA was precipitated with ethanol and resuspended in 10 µl of nuclease-free water.

**Circular RNA ligation**

RNA molecules having both a 5'-phosphoryl and 3'-hydroxyl end can be circularized by an intra-molecular ligation event. Circularization of RNA is a method that can permit the determination of 5' and 3' ends in unknown sequences using primers developed to known sequences near the ends. Circular RNAs have been used as templates for cDNA amplification reactions. Circularization was performed as follows: in a total volume of 400 µl, 15 µg of de-caped RNA was incubated whit 20 units of T4 RNA ligase

(5U/µl)(Epicentre Technologies, Madison WI), 40 µl 10X Buffer (330 m M Tris acetate pH 7.5, 660 mM potassium acetate, 100 mM magnesium acetate and 5 mM DTT) 20 units of RNasin® (40u/µl) (Promega, Madison, WI), 4 µl 10 mM ATP, 100 µg/ml acetylated BSA and nuclease-free water up to 400 µl. Incubate the reaction for 3 h at 37 °C. After extraction with phenol-chloroform, the RNA was precipitated with ethanol and resuspended in 10 µl of nuclease-free water.

**RNA-DNA amplification and visualization**

Polymerase chain reaction (PCR) is a powerful method that permits to generate millions of DNA copies starting from a limited amount of nucleic acid. While DNA is immediately suitable for such amplification, RNA must be reverse transcribed.

- **Reverse transcription**: The reverse transcription aims to synthesize the complementary DNA strand (cDNA) of each RNA molecules. The cDNA is then amplified by PCR. Moloney murine leukemia reverse transcriptase (M-MLV RT) (Promega, Madison, CA) was used for the common production of short fragments (up to 1-2 kb). RNA samples, mixed with 1 µl reverse primer (25 µM) and nuclease-free water up to 5 µl final volume, were first heated 10 min at 65°C in a T3000 Thermal Cycler (Biometra) to disrupt secondary structures. The elongation step was performed at 37°C in 1 h after the addition of 4 µl of 5X buffer (250 mM Tris-HCl, 375 mM KCl, 15 mM MgCl2 and 50mM DTT), 2 µl dNTPs (10 mM), 0.25 µl M-MLV RT (200 U/µl) and 3.75 µl steril and nuclease-free water. High quantities of longer cDNA fragments (1.5 – 6.0 kb) were synthesized using ImProm-II Reverse Transcriptase (Promega, Madison, CA). The denaturation step is identical to that described for M-MLV RT, and then 4 µl ImProm-II 5x-reaction buffer (250mM Tris-HCl (pH 8.3 at 25°C), 375mM KCl and 50mM DTT), 1.2 µl MgCl2 (25 mM), 1 µl dNTPs (10 mM), 0.5 µl RNasin Ribonuclease Inhibitor (40 U/µl), 1 µl Improm-II RT and 7.3 µl nuclease-free water were added. Elongation step is performed in 60 min at 42°C followed by RT inactivation at 70°C for 15 min. SuperScript ® III First-strand Synthesis Sysem (Invitrogen) was also used to synthesize RNAs target from 100bp to > 12kb.

RNA samples, was mixed with 1 µl of reverse primer (25 µM), 1 µl of 1m mM dNTPs and nuclease-free water up to 10 µl final volume, first heated 5 min at 65°C in a T3000 Thermal Cycler (Biometra), and then placed on ice for at least 1 min. The elongation step was performed at 50°C in 1 h followed by RT inactivation at 85 °C for 5 min after the addition of 2µl of 10X buffer (200 mM Tris-HCl, 500mM KCl), 4 µl of 25 mM $MgCl_2$, 1 µl of 0.1M DTT, 1 µl RNasin Ribonuclease Inhibitor (40 U/µl) (Promega, Madison, WI), and 1 µl SuperScript ® III RT (200 U/µl) .

- **Polymerase chain reaction (PCR)**: Three types of thermostable DNA polymerases synthesizing dsDNA were used in the presented experiments. Go Taq© Flexi DNA polymerase (Promega, Madison, CA) was used to amplify 1-2 kb fragments that didn't require high accuracy of copying, e.g. viruses detection or clones screening. Five µl of cDNA from RT step is mixed with 5 µl Go Taq® Flexi 5X buffer, 2.5 µl MgCl2 (25 mM), 0.75 µl dNTPs (10 mM), 1 µl of each primers (10 µM), 0.12 µl of Go Taq® DNA polymerase (5 U/µl) and sterile water up to a final volume of 25 µl. Pfu Ultra II Fusion Hotstart Polymerase (Agilent Technologies, Santa Clara, California, USA) was preferred when DNA fragments need to be subsequent cloned or sequenced. The reaction mix includes 2.5 µl PfuUltra II 10X reaction buffer (containing MgCl2), 1 µl of dNTP mix (25mM each dNTP), 1 µl of each forward and reverse primer (10 µM), 0.5 µl PfuUltra II Fusion HS DNA Polymerase (5 U/µl) and 5 µl cDNA from reverse transcription, nuclease-free water was added to a final volume of 25 µl.

  In order to amplified DNA template in the range of 0.2–2kb GoTaq® Long PCR Master Mix (Promega, Madison, CA) was also used. The reaction mix includes 12.5 µl of GoTaq® Long PCR Master Mix (50 units/ml of Taq DNA polymerase supplied in a proprietary reaction buffer (pH 8.5), 400µM dATP, 400µM dGTP, 400µM dCTP, 400µM dTTP, 3mM MgCl2), 1 µl of each forward and reverse primer (10 µM), 5 µl cDNA from reverse transcription and nuclease-free water was added to a final volume of 25 µl.

**Separation of DNA fragments on agarose gel by electrophoresis**

DNA fragments may be separated according to their size. For such purpose, gels are prepared by melting Multi Purpose agarose (Roche, Mannheim, Germany) in 1x-concentrated Tris-Borate-EDTA buffer (TBE, 89 mM Tris-borate, 8.9 mM boric acid and EDTA 2 mM). The agarose concentration can vary between 0.7 and 2.0%, depending on the fragments size. Using Biorad Power Pac 300 or Modell 1000/500 power supply (Biorad, Hercules, CA), DNA fragments are subsequently forced to migrate through the gel in TBE buffer 1x-concentrated towards the anode, as being negatively charged. Then the gel is stained on 200 ml of Ethidium bromide solution (0.1 mg/ml) that allows double strand DNA visualization under UV light. Using 1 kb or 100 bp DNA ladders (Promega, Madison, CA), the approximate size of the observed fragments can be determined.

**Purifications of nucleic acids**

DNA and RNA molecules may be purified for further manipulations.

When a single-type/length of DNA had to be selected among molecules of different sizes, all fragments were separated on agarose gel and the fragment of interest was selected and excised from the gel. DNA was subsequently extracted by using affinity columns of Wizard SV gel and PCR clean-up system (Promega, Madison, CA) and eluted with nuclease-free sterile water.

In order to purify DNA molecules the phenol-chloroform method is generally used. Equal volumes of hydrophobic phenol:chlorofom:isoamyl alcohol (25:24:1, pH 4.5) solution and DNA are mixed and centrifuged 15 min at 14,000 g at 4°C. The upper aqueous phase containing DNAs molecules is then precipitated with two volumes of 100% ethanol, 16 µl NaCl (5 M) and 1 µl glycogen (10 mg/ml) at -20°C during 20 min. After 20 min centrifugation at 14,000 x g at 4°C, the pellet is washed with 70% ethanol, dried and resuspended in nuclease-free sterile water.

The same protocol is also used for RNA purification, but in the precipitation step, glycogen and NaCl are replaced by Sodium Acetate (final concentration 150mM).

## A.3 Cloning

**Vectors**

- **pGEM®- T easy** (Promega, Madison, CA): this plasmid was used for common cloning or sequencing.
- **pBin61**: this plasmid of 12.9 kb is derived from the pBin19 plasmid and was used in agroinoculation experiments. pBin61 harbours a kanamycin resistance gene and contains a T-DNA between the left and right border sequences. Inside this T-DNA a 35S promoter sequence is followed by a multiple cloning site and a35S terminator sequence.
- **pJL89**: this binary vector has been used to produce CMV agroclones. It carries a kanamycin selectable marker.

**Enzyme digestion**

In order to obtain the desired final construct with the sequence of interest inserted in a specific vector, DNA has to be treated with restriction enzymes. Restriction enzymes recognize specific DNA sequences and cleave the double-strand to produce cohesive or protrusive extremities. One µg of template DNA is incubated with 0.4 µl of restriction enzyme (10 U/µl) and the appropriate restriction buffer at 37°C for 3 hours.

**Dephosphorylation**

In order to avoid self-ligation of the restricted plasmid and favor the insertion of the fragment of interest, phosphate groups of 5'-extremities of the linearized vector should be removed. Thus, the fragment to be inserted brings the only phosphate groups available and insertion is the only way to get circularization. One µg of

linearized vector is mixed with 1 µl of alkaline phosphatase from calf intestine (20 U/µl) (Roche), 2 µl dephosphorylation buffer 10x-concentrated (0.5 M Tris-HCl, 1 mM EDTA pH 8.5) and water to 20 µl final volume. The reaction is performed at 37°C for 1 hour.

**Ligation**

Ligation reaction was performed using the Rapid DNA ligation kit (Fermentas) according to the manufacturer's protocol. Usually, 1 µl of vector (50 ng/µl), 3 µl of insert (50 ng/µl), 3 µl Rapid DNA ligation buffer5X, 1 µl DNA ligase (1 U/µl) and nuclease-free sterile water up to 15 µl were used for each reaction, following incubation for at least 1 hour at room temperature. After phenol:chloroform purification and precipitation with ethanol, the ligation products are resuspended in 3 µl of nuclease-free sterile water and then used for electroporation.

For **pGEM®- T easy** ligation reaction was performed using DNA ligation kit (Promega, Madison, CA) according to the manufacturer's protocol. Usually, 1 µl of vector (50 ng/µl), 3 µl of insert (50 ng/µl), 5 µl Rapid DNA ligation buffer 2X, 1 µl DNA ligase (3 U/µl) and nuclease-free sterile water up to 10 µl were used for each reaction, following incubation for at least 1 hour at room temperature. After phenol:chloroform purification and precipitation with ethanol, the ligation products are resuspended in 3 µl of nuclease-free sterile water and then used for electroporation.

**Transformation of bacteria through electroporation**

One and half µl of plasmid DNA was added to 25/40 µl of electro-competent bacteria cells and the mixture was transferred to a special cuvette with two electrodes on its sides. Electroporation was carried out in a cell-electroporator (BioRad®, Hercules, CA) using the following settings: 125 µF capacitance, 200Ω (for *E. coli*) or 400Ω (for *A. tumefaciens*) resistance and 2.5V voltage. After electroporation, 500µl of LB medium was added and cells were left 30 min at 37°C (or 90 min at 28°C for *A. tumefaciens*) for

recovery. The transformed cells were spread onto solid LB medium containing the appropriate antibiotic.

**Plasmid extraction**

Transformed *E. coli* cells were grown overnight in 5 mL LB medium and plasmids were extracted by Wizard® Plus SV Minipreps DNA Purification system (Promega, Madison, CA) according to the manufacturer's protocol**.** Cell culture was briefly centrifuged, supernatant was discarded and pellet was resuspended in 250 µl resuspension solution (50 mM Tris-HCl pH7.5, 10 mM EDTA and 100µg/ml RNase A). 250 µl lysis solution (0.2M NaOH and 1% SDS) was added and homogenized by inverting tubes, then was added 10 µl of Alkaline Protease Solution and inverted 4 times to mix. Cell lysis was performed no longer than 5 min, then 350 µl of neutralization solution (0.759M CH3COOK, 2.12 M CH3COOH and 4.09M guanidine hydrochloride) was added and mixed by inverting. Tubes were centrifuged for 15 min at 15,000 x g, subsequently the supernatant was transferred to a Column into Collection Tube. Then centrifuged at top speed for 1 minute at room temperature, discarded flowthrough and reinserted Column into Collection Tube. 750 µl of Wash Solution (162.8 mM potassium acetate, 22.6 mM Tris-HCl pH7.5, 0.109 mM EDTA pH 8.0 and 35 ml of 95% ethanol was added) was added and centrifuged at top speed for 1 min. The flowthrough was discarded and the column reinserted into collection tube and repeated with 250 µl of Wash Solution. Centrifuged at top speed for 2 min at room temperature and discarded flowthrough. Centrifuged again for 1 min at top speed to remove completely Wash Solution and transfered the Spin Column to a new, sterile 1.5 ml microcentrifuge tube. Finally, plasmid was eluted in 100 µl $H_2O$ by centrifugation again for 1 min at top speed.

## A.4 Viral infection

**Mechanical inoculation onto test plant**

Leaves of host plants were mechanical rub-inoculated with sap extracts from leaves of kiwifruit plants with presumed viral infection. Leaves tissue were homogenized in 0.1 M Na-phosphate buffer, pH 7.5 containing 0.12% sodium sulphite and 5% polyvinylpyrrolidone in a mortar. Each leaf was dusted with Celite to promote mechanical lesions and facilitate penetration of transcribed RNAs into plant cells, and then gently rubbed with the inoculum.

**Agroinfiltration**

Agroinfiltration is an efficient methods for transient expression of gene in plants. Transformed *A. tumefaciens* cells were grown O/N at 28°C in 5 ml of liquid LB medium supplemented with rifampicin (50 µg/ml) and kanamycin (100 µg/ml). Bacteria were centrifuged for 10 min at 5,000 x g and pellet was resuspended in MA buffer (10 mM MgCl2, 200 µM acetosyringone), adjusting the OD600nm to 0.6. Bacteria were then incubated at room temperature for 3 h. Leaves of *Nicotiana benthamiana* plants were lightly incised with a scalpel and then infiltrated with the bacterial suspension using a syringe without the needle. Between 2 to 5 days post-infiltration or after symptoms appearance, leaves were harvested for analysis.

# Appendix C:

# Primers and accession numbers

**Table S 5.1:** Primer used on the characterization of AcLV

| Name Primer | Sequence (5'-3') | Position (nts) | Use |
|---|---|---|---|
| NGS CLO1_R | GAT GGT CAG TCT TAA TCTCC | 2,587-2,606 | Sanger |
| NGS CLO2_F | AGGAAT ATTGGA TCGGTTGTCG | 2,399-2,420 | |
| NGS CLO2_R | GTATACTCGAAAGTCTTCTCGG | 5,050-5,058 | |
| NGS CLO3_F | GTT TCG GAC GTA CTA GAT TTC G | 4,934-4,955 | |
| NGS CLO3_R | CTCCA AGA AGT AAC ACC ACC C | 7,525-7,545 | |
| NGS CLO4_F | ACC TAT AGT CAG CAA TTG TTC G | 7,438-7,459 | |
| NGS CLO4_R | GGAAATGCAGATGGTTTGTGAC | 10,032-10,053 | |
| NGS CLO5_F | CAG ACT TGT TCA GAG ATC ACG | 9,950-9,963 | |
| NGS CLO5_R | CCC ACC CTA CTG ATG TTC TC | 12,743-12,762 | |
| NGS CLO6_F | GGA TGA ACT ATT ACA AGT AGCG | 12,619-12,640 | |
| NGS CLO6_R | GAC ATA CAC GTC AACACA GG | 15,272-15,291 | |
| NGS CLO7_F | CTATATAGGTGATTGGGTTGCG | 15,145-15,166 | |
| NGS CLO7_R | ATAATCGCAAGAGTCGTTTTCG | 17,619-17,640 | |
| NGS CLO9_F | GCA GAA GAC CGG GAG GTC AC | 452-471 | |
| NGS CLO9_R | GCA TTC TTT CGG CGC AAT CTA CG | 1,691-1,713 | |
| NGS CLO10_F | GAA TGT CCG AAA AGT CCG ATT AGG | 3,224-3,247 | |
| NGS CLO10_R | CGT TCA ACC TAT TTA CTT CTG TCA TAC G | 3,977-4,004 | |
| NGS CLO11_F | GTGGTTAAGAAAATTCTGTCCAGC | 5,312-5,335 | |
| NGS CLO11_R | CCA AAC ATT CTC TTC CGG TCT TAC | 6,230-6,253 | |
| NGS CLO12_F | CTC GTG AGA AGG ATC ATA GCG G | 6,124-6,185 | |
| NGS CLO13_F | CAG TAT TCC CAC CAG ATC CTA TG | 8,053-8,075 | |
| NGS CLO13_R | GCA TCA GGA AAA TCT CGT CCA C | 8,921-8,942 | |
| NGS CLO15_R | CCTGATCGCGTTTATCTATGATGACG | 11,249-11,274 | |
| NGS CLO16_F | CAAGATCGGTGAGGAAAGTTAG | 11,182-11,203 | |
| NGS CLO17_R | CGA ACC ACC ATT CTT AGG AGT CG | 16,405-16,427 | |
| NGS CLO18_F | GAA GAC TTG TAA CGC TAC GTT G | 16,349-16,370 | |
| CLO 6773- R | TGGGTGTGGATGTTTCTTCTTATGC | 12,983-13,007 | |
| CLO 17265 F | GTTGTTTGGGGACTGGTTCAATGTC | 17,523-17,547 | |
| CLO 17491 R | CTCGTCCAGGATGTTCTCTAAAGG | 17,725-17,748 | |
| CLO 3847 F | TGTCGCTATCTAGAGGCAGG | 3,861-3,880 | |
| CLO 5357 R | TGACTTGCTCTGTAAATCTCCC | 5,350-5,371 | |
| HSP70F1 Kiwi | ATGACGATCATAGGTATYGACTACG | 12,769-12,793 | Detection |
| HSP70R1 Kiwi | CTTTGMGATGTRGTGTACTGAGAAGG | 13,204-13,229 | |
| HSP70F2 Kiwi | GTGAGTAYTATATAGTTTACGATTTCG | 13,343-13,369 | |
| HSP70R2 kiwi | ACTTTTCTATCTATACGRAACCTGG | 14,063-14,087 | |
| HSP70F3 Kiwi | AATTAYAAYGTCCGYTATCTCATAGC | 13,204-13,229 | |
| HSPR3 Kiwi | TCAGCGAGGATAGACTGAATATCCTTCTTCC | 14,493-14,523 | |
| CLO 111 circular R | CAGAAGCAGAACGGGAACTTTTGG | 108-131 | Circular RT-PCR |
| CLO 18653F | GAGCATCGTCATTCCACATG | 18,653-18,672 | |
| CLO 17535-17559 F | GTTGTTTGGGGACTGGTTCAATGTC | 17523 -17547 | Poly(A)Polymerase |

**Table S 5.2**: *Closteroviridae* abbreviations and accession number.

| Genus | Virus name | Accession # |
|---|---|---|
| *Ampelovirus* | *Grapevine leafroll-associated virus 1* (GLRaV-1) | NC016509 |
| | *Grapevine leafroll-associated virus 3* (GLRaV-3) | NC004667 |
| | *Grapevine leafroll-associated virus 4* (GLRaV-4) | NC016416 |
| | *Little cherry virus 2* (LChV-2) | NC005065 |
| | *Pineapple mealybug wilt-associated virus 1* (PMWaV-1) | AF414119 |
| | *Pineapple mealybug wilt-associated virus 2* (PMWaV-2) | AF283103 |
| | *Plum bark necrosis stem pitting-associated virus* (PBNSPaV) | NC009992 |
| *Closterovirus* | *Beet yellows virus* (BYV) | NC001598 |
| | *Carrot yellow leaf virus* (CYLV) | NC013007 |
| | *Citrus tristeza virus (*CTV) | NC001661 |
| | *Mint virus 1 (MV-1)* | AY792620 |
| | *Carnation necrotic fleck virus* (CNFV) | EU884443 |
| | *Raspberry leaf mottle virus* ( RLMV) | NC008585 |
| | *Grapevine leafroll-associated virus 2* (GLRaV-2) | DQ286725 |
| | Strawberry chlorotic fleck associated virus ( SCFaV) | DQ860839 |
| *Crinivirus* | *Bean yellow disorder virus* (BnYDV) | NC010560, EU191905 |
| | *Strawberry pallidosis-associated virus* (SPaV) | NC_005896 |
| | *Lettuce chlorosis virus* (LCV) | NC012909, FJ380119 |
| | *Lettuce infectious yellows virus* (LIYV) | NC003617, NC003618 |
| | *Tomato chlorosis virus* (ToCV) | KJ815045, AY903447 |
| | Diodia vein chlorosis virus (DVCV) | GQ225585, GQ376201 |
| | *Beet pseudo yellows virus* (BPYV) | NC005209, NC005210 |
| | *Strawberry pallidosis-associated virus* | NC005895, NC005896 |
| | *Blackberry yellow vein-associated virus* (BYVaV) | AY776334, AY776335 |
| | *Cucurbit yellow stunting disorder virus* (CYSDV) | AJ439690, AJ537493 |
| *Velarivirus* | *Cordyline virus 1* (CoV-1) | HM588723 |
| | *Little cherry virus 1* (LChV-1) | EU715989 |
| | *Grapevine leafroll-associated virus 7* (GLRaV-7) | HE588185 |
| Unassigned | Mint vein banding-associated virus ( MVBaV) | KJ572575 |
| Unclassified | Blueberry virus A (BVA) | KF007212 |
| | Carnation yellow fleck virus (CYFV) | NC022978 |
| | Persimmon Virus B variant1 (PeVBv1) | NC025967 |
| | Persimmon Virus B variant 2 (PeVBv2) | AB923925 |
| | *Grapevine leafroll-associated virus 5 (GLRaV-5)* | NC016081 |
| | Grapevine leafroll-associated virus 6 (GLRaV-6 | NC016417 |

| | | |
|---|---|---|
| Unclassified | Grapevine leafroll-associated virus 9 (GLRaV-9) | AY297819 |
| | Grapevine leafroll-associated virus 10 (*GLRaV-10)* | NC011702 |
| | Grapevine leafroll-associated virus Carn (GLRaCV*)* | FJ907331 |
| | Blackberry vein banding-associated virus (BVBaV) | NC022072 |
| | Grapevine rootstock stem lesion-associated virus (GRSLaV) | NC004724 |
| | Rose leaf rosette-associated virus(RLRaV) | NC024906 |
| | Mint-Like virus (Mint-like V) | NC024448 |
| | Cucurbit chlorotic yellows virus | JN641883, AB523789 |

**Table S 5.3**: *Totiviridae* and accession number

| **Genus** | **Virus name** | **Accession #** |
|---|---|---|
| *Victorivirus* | *Aspergillus foetidus slow virus 1* | CCD33023, CCD33024 |
| | *Helminthosporium victoriae virus 190S* | NP619669, NP619670 |
| | *Coniothyrium minitans RNA virus* | YP392466, YP392467 |
| | *Epichloe festucae virus 1* | CAK02787, CAK02788 |
| | *Sphaeropsis sapinea RNA virus 2* | NP047559, NP047560 |
| | *Gremmeniella abietina RNA virus L1* | NP624331, NP624332 |
| | *Magnaporthe oryzae virus 2* | YP001649205, YP001649206 |
| | *Magnaporthe oryzae virus 1* | YP122351, YP122352 |
| | *Tolypocladium cylindrosporum virus 1* | YP004089629, YP004089630 |
| | *Sphaeropsis sapinea RNA virus 1* | NP047557, NP047558 |
| | *Beauveria bassiana RNA virus 1* | CCC42234, CCC42235 |
| *Leishmaniavirus* | *Leishmania RNA virus 1 - 1* | NP041190, NP041191 |
| | *Leishmania RNA virus 1 - 4* | NP619652, NP619653 |
| | *Leishmania RNA virus 2 - 1* | NP043464, NP043465 |
| *Trichomonasvirus* | *Trichomonas vaginalis virus 3* | NP659389, NP659390 |
| | *Trichomonas vaginalis virus 4* | AED99797, AED99798 |
| | *Trichomonas vaginalis virus 2* | AED99809, AED99810 |
| | *Trichomonas vaginalis virus 1* | AED99819, AED99820 |
| *Totivirus* | *Saccharomyces cerevisiae virus L-A L1* | AAA50320, AAA50321 |
| | *Xanthophyllomyces dendrorhous virus L1A* | AFH09411, AFH09412 |
| | *Tuber aestivum virus 1* | ADQ54105, AAA50321 |
| | *Xanthophyllomyces dendrorhous virus L2* | AFH09415, AFH09416 |
| | *Xanthophyllomyces dendrorhous virus L1b* | AFH09413, AFH09414 |
| | *Botryotinia fuckeliana totivirus 1* | YP001109579, YP001109580 |
| *Giardiavirus* | *Giardia lambia virus* | DQ238861, DQ238862 |
| Unassigned | Gremmeniella abietina RNA virus L2 | YP044806, YP044807 |
| | Black raspberry virus F | YP001497150, YP001497151 |
| | Eimeria brunetti RNA virus 1 | NP108650, NP108651 |

# Appendix D:

# Viruses sequence

***Cucumber mosaic virus***

RNA1

GTTTTATTTACAAGAGCGTACGGTTCAATCCCTGCCTCCCCTGTAAAACTACCCTTTGAAAACTTCTCTCTCTTAATCTTT
TCTTTGTAATTCCTATGGCGACGTCCTCGTTCAACATCAATGAATTGGTAGCCTCCCACGGCGATAAAGGACTACTCGC
GACCGCCCTCGTTGATAAGACAGCTCATGAGCAGCTCGAGGAGCAATTACAGCATCAACGTAGAGGCCGTAAGGTCT
ACATCCGAAACGTTTTGGGTGTAAAGGATTCCGAGGTCATCCGGAATCGGTATGGAGGAAAGTACGACCTCCATCTTA
CCCAGCAGGAGTTTGCTCCCCACGGCCTAGCTGGTGCCCTCCGCTTGTGTGAAACTCTCGATTGTCTAGACTCTTTCCC
TTCATCAGGTCTGCGGCAGGACCTCGTCTTAGACTTCGGAGGAAGTTGGGTCACACATTACCTCCGCGGACATAATGT
ACATTGTTGTTCCCCCTGTCTGGGGATCCGCGATAAGATGCGCCACGCGGAACGTTTAATGAACATGCGCAAGATCAT
CTTGAACGATCCACAACAGTTCGATGGTCGACAGCCGGATTTCTGCACTCAACCGGCTGCGGATTGCAAAGTACAAGC
CCACTTTGCTATATCTATTCATGGAGGTTATGATATGGGCTTTAGAGGATTATGTGAAGCGATGAATGCTCACGGAAC
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CACAACGTGCTAGTTTCAGGGTACGGGTGCCCCCCACTTTCGTGGGGTCTCTAAAAGGAGACCAGGGTCGGCATGGC
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RNA2

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RNA3

GGGGGGATGTGCGCCATCCACTATCCTTCGCAAGACCCTTCCTCTATATAAGGAAGTTCATTTCATTTGGAGAGGGTA
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TCCGCACATTTTCTTTCAGTGTGTTAGATTTCCCGAGGCATGGCTTTCCAAGGTACCAGTAGGACTTTAACTCAACAGT
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ACCTAGGCCGGCATCACTGGATGCGCGCTGATAATGCTATTTCAGTCCGGCCCCTCGTTCCCGAAGTAACCCACGGTC
GTATTGCTTCCTTCTTTAAGTCTGGATATGATGTTGGTGAATTATGCTCAAAAGGATACATGAGTGTCCCTCAAGTGTT
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GTGGAGAAATCCACGCCAGTAGATTTACAAATCTCTGAGGCGCCTTTGAAACCATCTCCTAGGTTTCTTCGGAAGGAC
TTCGGTCCGTGTACCTCTAGCACAACGTGCTAGTTTCAGGGTACGGGTGCCCCCCCACTTTCGTGGGAGCCTCCAAAA
GGA

***Pelargonium zonate spot virus***

RNA 1

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AATGGCTGCTACTTCTTTTAATGTTCGTGACCTCATCAACTCCACTGGCGCTGATGCTATGGGAGTCAGGGGGTTGGTA
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GTAATCGTCGTTAAGACGTTGAGTGTTTACAAACACTCAAGATACGTATCCCGGGACTCTCTGCCGGGTGCAACAAGA
TGATCCTCATGAGGATTGTTAGATCTTGCGTATCGCTGAAGTAATTGAATGTGTTTGACACATTCTTTTCTTCGGTTTCT
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RNA 2

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ATGGCTGCTTTTACTTTCGAAAACTTTCTTTCTGGCGCCTACACAGGCCTTCCTATCGATAAATTCAGAGCTCTCGGTCT
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**Kiwifruit associated totivirus 1 (Totivirus)**

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**Actinidia latent virus (putative Closterovitus)**

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RB127 (poly(A) tailing clone):

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RB128 ( Circular RT-PCR):

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GCCATGATAGGTAATAATCCCCCTTCTAAAATCGCGTATTGTTTTTTAAGCGCGAAGTTTTGCATTTTCCCATGTGGAAT

GACGATGCTCA