VIRUS DEEP SEQUENCING: UNDERSTANDING THE UNKNOWN

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Abstract

Plant viruses are usually linked to plant or crop infection and disease, with many examples of viral infection causing social, environmental and economic damage in plants and crops described worldwide. Therefore, the choice of any particular management strategy which helps to avoid or reduce virus infection and disease in the field, needs to be specifically studied and evaluated according to the etiology of each pathogen, transmission and impact.

Field samples were collected from carrot crops during the period 2014-2016 across different regions and countries and were tested using real-time Polymerase Chain Reaction (RT-qPCR). Results revealed high levels of viral infection and showed the elevated presence of previously undescribed viruses such as *Carrot torrado virus1* (CaTV1) and *Carrot closterovirus 1* (CtCV1). Higher levels of infection found in year-round cultivation fields also indicated that this type of practice, widely used in the UK, might be detrimental and act in favour of virus infection. Further studies identified Cow Parsley and Hogweed as possible virus reservoirs of the tested viruses and identified the aphid species *Myzus persicae*, as the vector responsible of CaTV1 transmission.

Early detection of a pathogen from a field sample relies on the development and availability of a specific, sensitive and rapid diagnostic method. A RT-qPCR diagnostic assay has been developed and validated for the detection of CaTV1 according to the requirements established in the European Plant Protection Organization bulletin. Next Generation Sequencing (NGS) was used to study viral communities in carrots and a new viral sequence from the genus *Carlavirus* was identified in samples from the UK. By comparison with specific targeted diagnostics, this technique was shown to be a valuable approach for field surveillance and rapid detection of all the viruses present in a sample.

The use of more modern and sophisticated techniques such as NGS, has led to the discovery of high numbers of new viruses with unknown impact. As with CaTV1, many viruses have been found to not cause any acute disease in the infected plants. However, in this study some asymptomatic viruses, recently identified using NGS, were found to be more abundant in herbicide resistant than in wild-type sensitive black grass plants. Although no relationship between the presence of these viruses in the plants and the development of herbicide resistance could be established, the effects of asymptomatic infections are discussed.

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Chapter 1

Introduction

1.1 Introduction to plant viruses

1.1.1 Taxonomy

Virus particles are, with the exception of viroids, the smallest pathogens known and are composed of the genetic material, either RNA or DNA, and a protective structure. Most of the plant viruses known (90 %) are comprised of a RNA genome which might be single-(ss) or double-stranded (ds) and could code for up to 12 polypeptides. These polypeptides are involved in virus replication, structure, movement from cell to cell, transmission or gene silencing.

In 1966 an organisation named the "International Committee on Taxonomy of Viruses, ICTV" was created in order to develop an international nomenclature system for virus identification. The nomenclature of the new viruses is usually established according to the symptoms they cause, the major host or the host where they were initially described or the geographical location where they were firstly noticed. The last taxonomy report (ICTV 10^{th}), was released in early 2017 and is freely available online, where the new information is described and updated by international experts in the field. In this report, 4404 virus species were classified in 735 genera within 122 families and eight orders (ICTV online). The report also establishes the demarcating criteria for species differentiation and describes the main features and similarities of each taxonomic level. The major distinguishing criteria are based on virion properties such as shape and size, genome

organisation and replication strategy, properties of the proteins or other biological properties such as host range and transmission. In Figure 1.1 the current main families and genera of plant viruses described in the last report are shown.

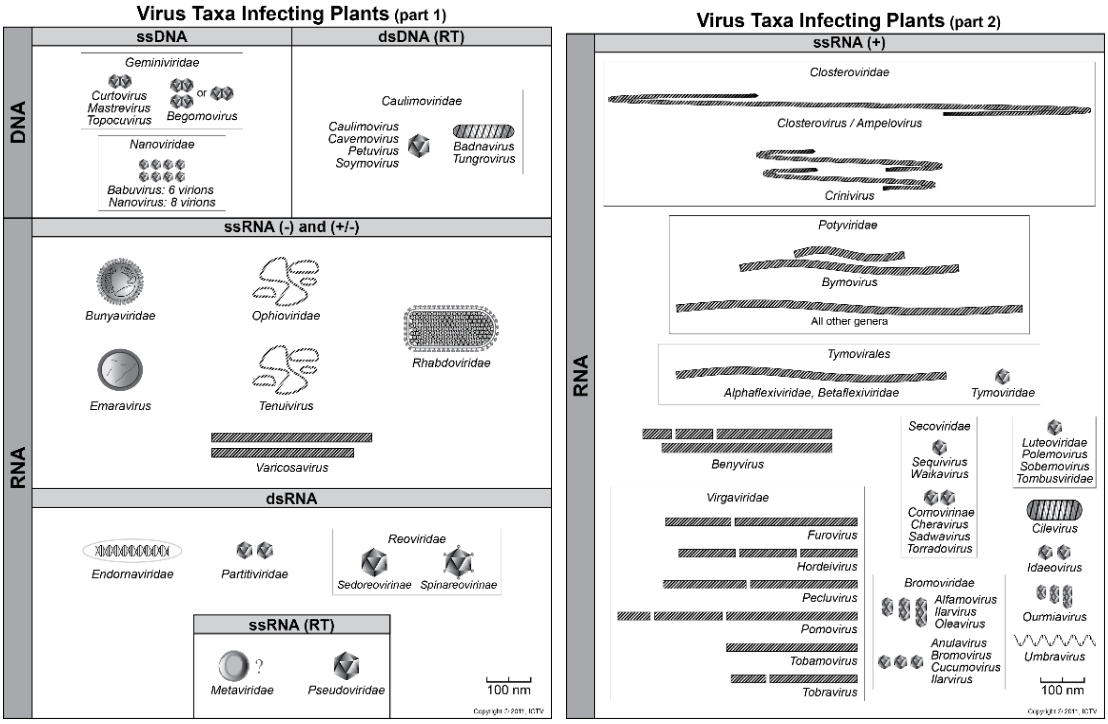


Figure 1.1: The families and genera of viruses infecting plants (ICTV 10th report).

1.1.2 Plant virus diversity

Virus populations are highly diverse and can become more adaptable to new environments and hosts as a result of genotypic variation and recombination. RNA recombinants are likely to arise driven by changes of environmental conditions, the crosses between viruses infecting different hosts or between viral and cellular RNAs among other factors (Bujarski, 2013). Previous studies have also indicated that when viruses get in contact with a new host, they could rapidly increase their infection rate by the appearance

of point mutations which enhance replication of the virus (Domingo, 2010). New strains of the virus could then arise and be responsible for an increase of the symptomatology and disease severity. For instance, the appearance of new strains of *Maize dwarf mosaic virus* (MDMV) led to heavy losses when it infected maize crops (*Zea mays*) in many areas of the USA (Walkey, 1991).

1.1.3 Virus transmission

Plant viruses need to be spread from one susceptible host to another for their own survival. There are numerous ways in which a virus can be transmitted and spread within a crop. Some viruses for instance, are transmitted vertically, in seeds or via pollen and spread by wind. However, most plant viruses are transmitted from one host to the other by vectors that can introduce viruses across cell walls. The most important group of vectors known are insects (over 400 insect species have been reported to transmit viruses), but they can also be transmitted by nematodes and fungi. Some of the most common insect vectors are aphids, for example *Myzus persicae,* known as the green peach aphid, which is worldwide distributed and has been reported to transmit more than 100 plant virus diseases (Harrison, 1958; Kanavaki *et al*., 2006). Whiteflies such as *Bemisia tabaci* or *Trialeurodes vaporariorum,* are also common vectors known to transmit at least 115 virus species from different genera (Monger, 2009). Plant viruses are usually highly specific for their transmission vectors and a virus transmitted by a certain vector type (e.g. aphids, nematodes…) is not usually transmitted by another.

1.2 Losses caused by plant viruses

Viral infection tends to reduce crop production leading to a quality or yield reduction and social and economic losses every year (Table 1.1). Plant viruses and phytoplasmas were identified as the cause of 51 % of the emerging infectious diseases of plants worldwide during the period of 1996-2002 according to data published in the ProMED (Program for Monitoring Emerging Diseases) database (Rodoni, 2009). Losses ranging from 25-50 % have been reported depending on the crop and season (Oerke *et al*., 1994). There are countries where a large proportion of the population is highly dependent on the production of a single or few crops and where an epidemic would have devastating consequences for the population. For instance, in late 1990, Cassava (*Manihot esculenta*) production was reduced by 80-90 % due to infection with Cassava Mosaic Virus Disease and many farmers suspended its cultivation in East Africa where 50 % of the current worldwide Cassava production takes place (Vurro *et al*., 2010). That led to huge social and economic losses, which were estimated around 60 million dollars. In Asia, *Rice tungro bacilliform virus* (RTBV) leads to an estimated economic loss of 1.5 billion dollars annually in rice (Hull 2002) and *Cocoa swollen shoot virus* (CSSV) is estimated to cause an annual loss of 28 million dollars (Bowers *et al.,* 2001). *Barley yellow dwarf virus* (BYDV) is distributed worldwide and has a wide host range infecting more than 150 species in the *Poaceae* family, including wheat (*Triticum* spp.), barley (*Hordeum vulgare* L.) or corn (*Zea mays* L.), all of them being major food sources for many countries (Trebicki *et al*., 2015).

In some cases, visual symptoms do not correlate with yield losses. For instance, *Tobacco ringspot virus* (TRSV) can reduce germination capacity of the germ tube in soybean (*Glycine max*). The result is a failure to fertilise the egg which leads to a reduction of the final yield without obvious disease symptoms in the foliage of the infected plants (Yang & Hamilton, 1974).

Table 1.1: Examples of economic losses per year caused by infection with different viruses in different crops worldwide.

Crop	Virus	Countries	Loss/Year
Rice	Tungro Ragged stunt Hoja Blanca	SE Asia SE Asia S. and C. America	$\$1.5 \times 10^9$ $\$1.4 \times 10^8$ $\$9.0 \times 10^6$
Barley	Barley yellow dwarf	UK	$£6 \times 10^6$
Wheat	Barley yellow dwarf	UK	$£5 \times 10^6$
Potato	Potato leafroll Potato virus Y Potato virus X	UK	$£3\text{-}5 \times 10^7$
Sugar beet	Beet yellows Beet mild yellows	UK	$£5\text{-}50 \times 10^6$
Citrus	Citrus tristeza	Worldwide	$£9\text{-}24 \times 10^6$
Cassava	African cassava mosaic	Africa	$\$2 \times 10^9$
Many crops	Tomato spotted wild	Worldwide	$\$1 \times 10^9$
Cocoa	Cocoa swollen shoot	Ghana	1.9×10^8 trees

*Data from Comparative Plant Virology (Hull, 2002).

1.3 Control measures for plant protection

The choice of management practices for a particular disease is highly specific on the nature of the virus and how it is transmitted and spread within the crop. It needs to also be compatible with the cultural practices essential for crop production, simple, effective and inexpensive, within the limited resources of the farmers involved. Sometimes the use of the same control strategy in the same crop might differ in their effectiveness and, in some cases, the use of different measures is recommended in order to achieve an integrated and harmonized manner to maximize the benefits of each method (integrated approaches).

1.3.1 Conventional practices

Contrary to fungal or bacterial diseases, plant viral diseases are not amenable to control by direct methods such as with the use of fungicides or bactericides. However, there are several indirect and conventional measures which are usually targeted to reduce sources of virus infection, to limit its spread by the vector or to reduce the effects of infection. An important approach is to decrease or eliminate virus reservoirs, usually wild species which harbour many viruses that can be later transmitted to the field, and spray them with selective herbicides. Sowing virus-free tested seeds, or adjusting seedling/planting times based on vector migration to avoid peak populations are other common techniques used to avoid infection and control virus spread. The growing of the same crop in sequential seasons is not usually recommended as some pathogens can remain in the soil (either as infected roots or in viruliferous vectors) for a certain time after harvesting. Also, it can result in a lack of certain nutrients in the soil leading to quality and yield reduction. Crop rotation, the growing of different types of crops in succession on the same land, has been widely used over years to avoid these problems (Jones *et al*., 2006). The use of crop protection products in the plants might be undesirable in particular occasions as they can encourage vector populations to move rapidly between plants or kill beneficial organisms that help with

vector suppression. Cleaning and decontaminating machinery or avoiding overlapping sowing/planting periods might also be beneficial measures to avoid infection between fields or seasons (Hull, 2002). For instance, strict hygiene measures are currently the best strategy to prevent the spread of *Pepino mosaic virus* (PepMV) in crop production.

1.3.2 Biosecurity

Globalization of agriculture and the intercontinental movement of plants and products have meant the introduction and cultivation of plants and crops out of their original habitat, where they are poorly equipped to resist the different natural conditions and the pathogenic organisms that may be resident there. For instance, an estimated 5.000 foreign plant species have been reported in the USA, many of them imported as ornamentals for cultivation but now established in the ecosystem (Pimentel *et al*., 2004). One example is the Cassava crop, with millions of tons produced every year and mostly produced in food-deficit countries. This crop has been found to be usually infected in East Africa with *Cassava brown streak virus* (CBSV) and *Ugandan cassava brown streak virus* (UCBSV) leading to Cassava brown streak disease (CBSD). The disease has been reported to be the most important Cassava viral disease and a threat to crop security as infection leads to reduced yield and in turn, economic loss in the area (Vurro *et al*., 2010, Basavaprabhu *et al*., 2015,). However, these viruses, and consequently, CBSD, have not been reported in South America, the centre of origin of the crop (Thresh, 2005). This has resulted in the implementation of diverse control strategies to enhance food biosecurity; risks assessment, regular inspections or quarantine actions have been established in order to minimize the impact of these new pests and pathogens in their new habitats.

1.3.3 Protecting the plant from infection

Inoculation of the plants with a mild strain of a virus, a phenomenon known as cross-protection, can result in the plant being protected against a more severe strain (Fraser, 1998). One example is *Citrus tristeza virus* (CTV), with more than 8 million trees inoculated in Brazil by 1980 with a mild isolate of the virus (Muller *et al*., 2004). A mild isolate of PepMV was also used to protect tomatoes against more aggressive strains causing severe symptoms (Hasiów-Jaroszewska *et al*., 2014). However, this technique needs to be continuously assessed and is not always recommended as a general practice. Mild strains can reduce yields by 5-10 %, recombine with other viruses present in the host or evolve to a more severe strain in some plants. Resistance against the vectors can be sometimes limited as it does not protect against the virus. Plant breeding for resistance, consisting of the genetic improvement of the crop to make it disease resistant, has been used against sweet potato virus disease (SPVD) (Ngailo *et al*., 2013). Also, resistance to *Rice yellow mottle virus* (RYMV) in rice was achieved in Africa using varieties containing a transgene encoding the RNA-dependent RNA polymerase of RYMV (Pinto *et al*., 1999).

1.3.4 Asymptomatic viruses and beneficial effects of virus infection

In recent years there has been a significant increase in the number of viruses discovered, showing the limited knowledge of the natural "viral biota" in a particular host. Many of these viruses have been identified using novel sequencing techniques and have not been associated with the development of disease symptoms, but even limited replication and accumulation of virus particles must provide a fitness cost to the plants. This fact has resulted in an increasing interest in the study of asymptomatic viruses and some researchers have even postulated that, although viruses have always been considered as natural pathogens, infection could also provide beneficial effects to their hosts (Roosinck, 2011, Roosinck, 2013). Although not fully understood, these effects could range from obligate

mutualisms where survival of the host depends on the presence of the virus, to beneficial effects under specific abiotic stresses. For instance, *Cucumber mosaic virus* (CMV) was seen to improve drought tolerance of various host plants (Xu *et al.*, 2008) (Figure 1.2). Panic grass (*Panicum*) has also been seen to be able to grow in soil temperatures up to 65 °C when the fungus *Curvularia protuberata* infected by *Curvularia thermal tolerance virus* are present (Márquez *et al*., 2007). The *White clover cryptic virus* (WCCV) genome encodes a coat protein that can help with reducing root nodulation by activating the plant's innate immune response in a model legume (*Lotus japonicus)* (Nakatsukasa-Akune *et al.*, 2005).



Figure 1.2: Infection with *Cucumber mosaic virus* (CMV) can improve drought tolerance of infected hosts (Xu *et al*., 2008).

As mentioned, cross-protection can also be used against more severe strains of a pathogen. All these examples provide an unexplored new approach of plant viruses where these advantages could be used to enhance and improve crop production under certain conditions.

1.4 The importance of diagnosis in plant virology

Early detection and prevention are two key approaches that can be used to minimize disease damage in crops and, as a consequence, maximize productivity and agricultural sustainability. Usually recognition of the problem, collection of epidemiological data and the development of a diagnostic method require time. Sometimes even the identification of a pathogen responsible for a new or unusual viral disease can take weeks or even years using traditional methods. For instance, the identification of the viruses responsible for lettuce big-vein disease, *Lettuce big-vein virus* (LBVV) and *Mirafiori lettuce virus* (MiLV), took over 60 years since the first report in 1934 (Lot *et al*., 2002). Identification of a new virus often requires confirmation with two or more different methods, especially for unexpected or novel viruses.

Diagnostics based on symptomatology of the infected plants might be sometimes difficult and inexact: different viruses might lead to similar symptoms and different symptoms are caused by the same virus in different crops, or different conditions (abiotic stresses, single or mixed infection etc.). In addition, the detection and control methods developed after identification need to be readily accepted and validated for good quality assurance. The European Plant Protection Organization (EPPO) bulletin established a series of requirements desirable for a detection method to be effective and implemented (OEPP/EPPO Bulletin PM7/98, 2014). These are usually related to the sensitivity, specificity and accuracy of the method as well as repeatability and reproducibility when this is performed several times and by different users.

1.4.1 Traditional methods

Methods such as mechanical inoculation to indicator plants or transmission electron microscopy (TEM) have been used for decades for pathogen identification. Non-

specific methods such as host plant inoculation (biology) can help us to confirm the presence of the virus in a sample but they usually take a few weeks and sometimes results are not conclusive as the same symptoms can be caused by different viruses or the plants can present different symptoms when they are grown out from the field. Diagnosis based on visual inspection of symptoms also requires a high level of expertise by specialists in the area and, since it is time consuming, the number of samples inspected using this method is usually low. Furthermore, many viruses are latent or asymptomatic which makes them more difficult to be recognised, requiring confirmation using a second method.

1.4.2 Serological methods

Serological detection techniques use specific antibodies developed which interact with a specific region of a protein (antigen) of the pathogen. ELISA has been postulated as the most valued serological technique since its introduction (Clark and Adams, 1977). Briefly, it is based on the use of specific antibodies fixed on the surface of a plate which recognise antigens present in the sample (usually the coat proteins of the viruses), with an enzyme linked to the antibodies. The plant virus antibodies can be synthesized and bind their targets specifically at various levels: isolates, species, strains, sub-groups and genera. The technique resulted in a huge improvement in the diagnostic arena raising the possibility of detecting viruses specifically at low titres and its rapidness and easy interpretation of results made it suitable for the screening of large batches of samples in short periods of time. Many variations of ELISA have been developed since then (Clark and Bar-Joseph, 1984; Van Regenmortel, 1993) and it has also been used in combination with electron microscopy (ISEM: immunoabsorbent electron microscopy, SSEM: serologically specific electron microscopy) providing a rapid diagnosis and features of the virus at the same time. Tissue blot immunoassay (TBIA), uses the same principle of ELISA but the assay is performed on nitrocellulose/nylon membranes. Other methods are Lateral Flow Devices,

which are simple and inexpensive and widely used for field testing. The Dot Blot technique represents a simplification of the western blot method where samples are spotted onto a nylon/nitrocellulose matrix and proteins of the virus can be detected using antibodies (usually with an enzyme or a fluorescent reagent) (Harper *et al*., 1995).

1.4.3 Nucleic-acid based methods

The use of methods which detect viral RNA/DNA have increased significantly during the past decades in part due to their speed, increased sensitivity and specificity in comparison with other techniques. Polymerase chain reaction (PCR) has rapidly become the most commonly used molecular technique since its first publication (Mullis, 1986). It is based on the exponential amplification of the target viral region using the DNA polymerase and thermal cycling. Advanced PCR methods such as reverse-transcriptase PCR (RT-PCR), where an additional step is performed to produce a cDNA strand complementary to the virus, or multiplex PCR where multiple targets can be detected simultaneously have been developed (Bertolini *et al*., 2001). In recent years, a variation of the technique was introduced, known as real-time or quantitative PCR (qPCR), where the detection of the PCR products is performed via the generation of fluorescence. A commonly used chemistry for this method is TaqMan™, based on the use of an extra primer marked with a fluorescence dye and quencher (probe) which recognises an internal region of the PCR product, the 5'-3' exonuclease activity of *Taq* polymerase and Fluorescent Resonance Energy Transfer (FRET) phenomenon (Holland *et al*., 1991; Heid *et al*., 1996). At the end of each experiment, the amount of fluorescent signal is proportional to the amount of the PCR product amplified and this can be monitored in real time (Higuchi *et al*., 1993). TaqMan™ is currently considered the gold standard technique in diagnostic laboratories for the detection of plant pathogens, as it allows screening of large batches of samples, target quantification and monitoring of the results in shorter periods of time (Alemu, 2014).

Loop-mediated isothermal amplification (LAMP) has been postulated as a new approach for on-site testing for plant viruses due to its simplicity and speed (Boonham *et al*., 2013). Similar to real-time PCR however three pairs of primers are used and amplification is performed without thermal cycling using a strand-displacing DNA polymerase at a single temperature of around 65 °C. Nucleic acid based Dot blots can also be used for detecting viral nucleic acids using labelled probes, as a variation of northern blot and southern blot methods and it has been used in combination with RT-PCR for the detection of six potyviruses (Hsu *et al*., 2005). Microarrays, consisting of the immobilization of specific oligonucleotide sequences onto a solid surface, were introduced as a multi-target hybridisation method for the simultaneous detection of multiple pathogens in a single assay (Boonham *et al*., 2007).

In recent years, Next Generation Sequencing (NGS) has revolutionised the sequencing picture and has become a very popular method to obtain whole virus sequences in short periods of time. This new approach represents a variation of the classic Sanger sequencing as it allows massively parallel sequencing of samples in a single run (Ronaghi *et al*., 1998; Ronaghi, 2001; Fakruddin *et al*., 2013). NGS platforms developed by Illumina (US) are currently the most used systems. The development of this technique has resulted in the sequencing of whole viral genomes in shorts period of time, characterization of virus populations in various environments and identification of viral communities in a particular host (Kreuze *et al*., 2009; Coetzee *et al*., 2010; Masart *et al*., 2014). The success of NGS has been accompanied by the development of powerful bioinformatics tools which allow a rapid analysis of the huge amount of data generated.

1.4.4 Discovery of novel viruses

In the last decades there has been an increase in the number of new plant viruses discovered, driven in part by the international movement of plant products and the development of new sequencing technologies such as NGS. The use of this technique has established the little information known about the viral biota of different populations, estimated now to be much larger than initial predictions. In the last few years, more than 100 new plant viruses from different genera, families and different hosts have been identified, providing us with a better picture of the world of plant virology (Kreuze *et al*., 2009; Al Rwahnih *et al*., 2009; Sabbadin *et al*., 2017). NGS has also helped to identify viruses responsible for a disease, when these could not be identified using routine diagnostic methods such as PCR. For instance, in 2012, NGS was used to identify *Maize chlorotic mottle virus* (MCMV), and *Sugarcane mosaic virus* (SCMV), two new viruses causing maize lethal necrosis in Kenya (Adams *et al*., 2013). Also, previously undescribed viruses (Carrot closterovirus 1, *Carrot torrado virus 1* and two new Betaflexiviruses) were found in 2013 studying the causes of internal necrosis in carrots (Adams *et al*., 2014). After initial identification, the presence of the new viruses in the samples is usually confirmed using PCR.

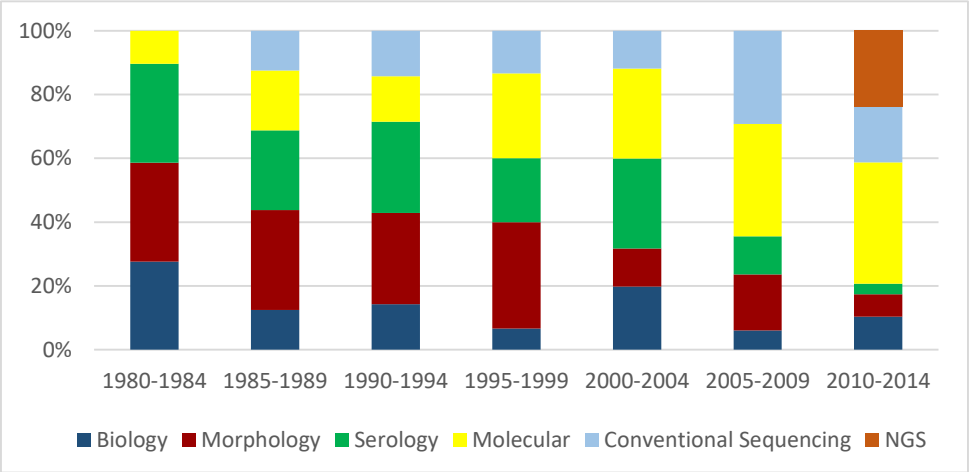


Figure 1.3: Percentages for each family of methods of first detections and new discoveries in the UK during the period 1980 – 2014 (Fox and Mumford, 2017).

In Figure 1.3 is represented for each family of methods the percentages of new discoveries and first detections in the UK in the period 1980-2014. Although between 1980 and 1999, the percentages of new identifications using traditional methods such as biology (host plant inoculation) and morphology (electron microscopy) rounded 40-58 %, this percentage decreased in the following years with the advent of more modern techniques such as qPCR or sequencing. A decrease in the number of new findings and detections is observed using ELISA during the last periods probably due to a major use of RT-qPCR. A potential reason might be that PCR primers are easier and quicker to design and synthesize than novel antibodies for ELISA. Also the set-up cost of new antibodies for ELISA is considerably higher compared with primer synthesis. Finally, the use of RT-qPCR is of more generic applicability in diagnostic laboratories as it can be used in diagnostics for fungi and bacteria and results can be determined in less than 2 hours. However, PCR also has some limitations: it depends on the efficacy of the nucleic-acid extraction, the quality and specificity of the oligonucleotides used and the absence of inhibitors in the sample assay (Fang *et al*., 2015). The rapidness (~ 30-40 min), and simplicity of LAMP, are making this technique a new approach for on-site testing and suitable in countries with limited resources. Although microarrays can be used to detect multiple targets simultaneously, they have not been adopted for routine use so far, probably due to the advent of new sequencing technologies in recent years. The period 2010-2014 in Figure 1.3 is characterized by the appearance of NGS, when 25 % of the new identifications took place using this technique. Contrary to PCR and ELISA, where previous information about the target is needed, NGS does not rely on previous knowledge of the pathogen and (Adams *et al.,* 2009, Barba *et al*., 2014). The continous development of new and easier platforms and the decrease of sequencing costs per run, are making this technique viable as the next technology to become adopted as a routine diagnostic tool (Studholme *et al*., 2011). However, NGS requires comparison with genome sequences currently available in databases, resulting in a limitation in the analysis.

1.5 Carrot crop and main diseases

Carrots (*Daucus carota L.*) are included in the top ten of the most economically important vegetables in the world in terms of production and market value. The main carrot producing countries are China, Russia and the US and in the UK, one of the main carrot producers within the EU, approx. 700000 tons are produced each year (Eurostat website - agricultural production). Although this crop is less intensively produced than other crops such as wheat, rice or potatoes, it fulfils essential nutritional requirements for the population as it is the main source of Vitamin A in the diet and rich in β-carotene which acts as an antioxidant. Carrots belong to the *Apiaceae* family, previously known as *Umbelliferae*, with other known and consumed crops such as celery (*Apium graveolens*), parsnip (*Pastinaca sativa*) and parsley (*Petroselinum crispum*). The Nantes type is the most widely cultivated in the world (50 % of the volume), followed by Bolero and Chantennay but it usually depends on the preferences and climate conditions of each region.

Other well-known diseases caused by different pathogens are: Alternaria leaf blight (*Alternaria dauci*), considered one of the most economically important diseases of carrots causing brown to dead spots on the infected leaves (Farrar *et al*., 2004). The use of clean seeds might help prevent the disease and, in case of infection, the use of some fungicides is recommended. However, prevention has been the best strategy to control the disease so far. Bacterial leaf blight (*Xanthomonas hortorum*) is a well-known bacterial carrot disease with a worldwide distribution. Similar to Alternaria leaf blight, yellow spots and irregular shaped leaves tend to appear on the infected leaves. The use of virus-free seed or the application of appropriate bactericides might help to prevent or control the disease respectively along with the use of decontaminated machinery and avoidance of continuous cropping. Finally, Cavity spot is a widely distributed disease, caused by different species of oomycetes, including *Pythium violae, P. sulcalum and P. sylvaticum* which are usually in the soil and infect carrot roots (McDonald, 1994). Typical symptoms are the appearance

of deep and dark cavities in the crop, making the carrots not acceptable for the fresh market. Although there is no effective management strategy to control the disease, it is recommended not to plant in soils with a history of the disease or with a high clay content.

1.5.1 Carrot viruses

Currently more than 30 viruses are known to affect carrot crops worldwide and examples of quality reduction and yield losses have been described worldwide (Latham and Jones 2004). Although the presence of several viruses affecting carrot crops in the UK is extensively studied in chapter 2 in previous limited surveys was found that the main viruses affecting carrot crops in the UK were Parsnip yellow fleck virus (PYFV) and the Carrot mottle dwarf complex (CMD) consisting of *Carrot mottle virus* (CMoV), *Carrot red leaf virus* (CTRLV) and Carrot red leaf associated virus (CRLaV). Both have been the primary focus of attention in carrot virus research with significant variation of incidence from season to season.

Carrot mottle dwarf disease

The Carrot mottle dwarf disease (CMD) has been reported in Australia (Stubbs, 1948), Japan, USA and Europe (Watson 1960). The "mottle dwarf" disease was described in 1948 by Stubbs (1948). Carrots are often infected with *Carrot red leaf virus*, (CtRLV) and *Carrot mottle virus* (CMoV) and the association of both viruses results in an intensification of the symptoms and development of the disease: reddening, yellowing and stunting. However, in experimental studies, single infections indicated the appearance of mild symptoms in the plants. Usually, infection of older carrot produces milder symptoms than in young carrots.

Regarding the transmission, these viruses are transmitted by the willow carrot aphid *Cavariella aegopodii* in a persistent and circulative manner (Elnagar and Murant, 1978; Watson *et al*, 1964). CMoV cannot be vectored by the aphid unless is accompanied by CtRLV (Watson *et al*., 1964). To do this, CMoV is packaged in CtRLV coat protein. CRLaV was found in 2003 in the UK although it was firstly discovered in California in 1998 with CtRLV and CMoV (Watson *et al*., 1998). It depends also on CtRLV for cell to cell movement and aphid transmission although it can replicate itself independently. No disease symptoms have been reported in the crop due to infection with this virus.



Figure 1.4. Symptoms developed in carrot plants due to infection with Carrot mottle dwarf disease (CMD).

Parsnip yellow fleck virus

Parnsip yellow fleck virus (PYFV) is a plant picorna-like virus within the *Secoviridae* family and is recognised as an early season threat in carrot crops. Early infection usually results in stunted plants and death of many of them and later infection is usually characterised by yellow flecks in the foliage. Transmission depends on the presence of *Anthriscus yellow virus* (AYV) as a helper virus and is carried out also by the willow-carrot aphid in a semi-persistent manner. Previous studies suggested that the main source of PYFV in carrots is associated with the presence of the weed species of cow parsley (*Anthriscus sylvestris*). However, as carrots are not a host for AYV they cannot subsequently transmitted within the crop.

Novel carrot viruses

Novel carrot viruses were found in a Next Generation Sequencing study in 2013 when the causes of internal necrosis in carrots were being investigated (Adams *et al*., 2014). As these were novel viruses identified by sequencing methods, no information was described or available about the symptoms they cause in the infected plants, host range or possible transmission vectors. The viruses identified in the study are the following: a new torradovirus tentatively named *Carrot torrado virus 1* (CaTV1), Carrot Closterovirus 1 (CtCV1) and two new Betaflexiviruses from the genus *Chordovirus*, *Carrot chordovirus 1* and *Carrot Chordovirus 2* (CtChV1 and CtChV2).

1.6 Black grass novel viruses

Black grass (*Alopecurus myosuroides*) is an annual weed highly associated with yield reduction in cereal crops. Herbicides have always been the primary control method but in the last two decades herbicide resistance has been confirmed in the North of Europe and in the UK (Hull *et al*., 2014). This fact led to the study and development of alternative control measures or new herbicides with different modes of action. A NGS study carried out by Sabaddin *et al*., 2016, in order to study viral communities in black grass, identified novel viruses in different herbicide resistant and sentitive populations around the UK. These novel viruses were: Alopecurus myosuroides partitivirus 1 (AMPV1) and Alopecurus myosuroides partitivirus 2 (AMPV2), within the *Partitiviridae* family, and Alopecurus myosuroides varicosavirus 1 (AMVV1) in the *Varicosavirus* genus. No relation was found between the presence of the novel viruses and the development of symptoms. Furthermore, as these are sequencing identified viruses, information related to symptomatology, host range or transmission remains still unknown. That is why AMPV1, AMPV2, and AMVV1

are currently subject of further investigations in order to understand the consequences of infection in black grass.

1.7 Aims of the study

The current situation of the carrot crop in the UK and the need of a deeper understanding of new asymptomatic viruses within the crop and in black grass populations, established the following aims for this thesis:

(a) to assess the incidence of known and new sequencing identified carrot viruses in the UK (CMoV, CtRLV, CtRLVaRNA, CYLV, CaTV1 and CtCV1). Identify possible virus reservoirs and transmission vectors of the novel viruses CaTV1, CYLV and CtCV1.

(b) to investigate viral communities present in carrot crops in different regions and establish potential sources of infection using Next Generation Sequencing (NGS).

(c) to characterize and develop a new qPCR diagnostic method for the detection of a novel virus affecting carrots, *Carrot torrado virus1* (CaTV1). This virus was found in asymptomatic carrot samples and it has not been related to the development of any symptom so far.

(d) to study the role of asymptomatic novel viruses affecting black grass populations in the UK and establish their relation with the development of herbicide resistance.

Chapter 2

Epidemiology of carrot viruses

In this chapter, carrot and weed samples were collected from carrot fields in the UK (2014, 2015) and France (2016) and tested in order to assess the presence of known and novel viruses. The sampling in France was carried out during a three months placement at the Institut National de la Recherche Agronomique (INRA) in Bordeaux (France) under the supervision of Dr. Thierry Candresse. Weed samples, provided by the Warwick Crop Centre, were tested to look at possible virus reservoirs and were used to study virus diversity.

The PhD candidate collected the carrot and weeds samples from the fields in the UK and France and extracted and tested all the samples using RT-qPCR. Testing of the weed samples from Warwick was carried out by the Virology group at FERA. The candidate performed virus diversity studies using Next Generation Sequencing and analysed the data.

Abstract

Field surveys were carried out across the UK and France during the period 2014-2016 in order to assess the incidence of known and novel carrot viruses using RT-qPCR. Results indicated different levels of infection between years and also between regions. In the UK, in addition to previously described viruses such as the viruses of the Carrot mottle dwarf complex (CMD) or *Carrot yellow leaf virus* (CYLV), sequencing identified viruses, *Carrot torrado virus1* (CaTV1) and Carrot Closterovirus 1 (CtCV1), were also found at high percentages. In 2015, levels of infection up to 100 % were found with the viruses associated with CMD. Field surveys carried out in France resulted in the first finding of CaTV1 outside the UK in a single field where carrots were cultivated year round and no vector control measures were taken. Higher percentages of infection found in fields with year-round cultivation of carrots suggested that this type of practise could be detrimental. Weed testing carried out to look at possible virus reservoirs did not result in any positive results for CaTV1. However, CYLV and CtCV1 were found at high prevalence, being Cow Parsley (*Anthriscus sylvestris*) and Hogweed (*Heracleum sphondylium*) the predominant hosts. Next Generation Sequencing analysis revealed that these hosts can also be virus reservoirs of two new sequencing-identified viruses from the genus *Chordovirus*.

2.1 Introduction

Currently more than 30 viruses are known to affect carrot crops worldwide (Brunt *et al*., 2017). Limited surveys and previous results indicated that the main viruses affecting carrot crops in the UK were *Parsnip yellow fleck virus* (PYFV) and the Carrot mottle dwarf complex (CMD) consisting of *Carrot mottle virus* (CMoV), *Carrot red leaf virus* (CtRLV) and Carrot red leaf associated RNA (CtRLaVRNA). PYFV is a recognised early season threat in carrot crops causing a wide range of symptoms in the infected plants (Morgan, 2004; van Dijk *et al*., 1985); early infection usually results in stunted plants and seedling death whilst late infection is characterised by yellow flecks in the foliage. Transmission is semi-persistent (Elnagar *et al*., 1976a, 1976b) and depends on the presence of a helper virus, *Anthriscus yellows virus* (AYV) and the vector, *Cavariella aegopodii,* (willow-carrot aphid). Infection with the viruses of the CMD complex can also result in the presence of disease symptoms such as reddening, yellowing and stunting and has been reported worldwide (Gungoosingh *et al*., 2009). It is also transmitted by the willow carrot aphid in a persistent and circulative manner but CMoV cannot be vectored by the aphid unless it is accompanied by CtRLV (Elnagar *et al*., 1978). Recently, transmission by the aphid species *Myzus persicae* was also reported (Naseen *et al*., 2016). PYFV and CMD have been the primary focus of attention in carrot virus research for a number of years and significant variation in incidence has been reported from season to season (Fox *et al*., 2017).

Understanding the scale and diversity of the viral biota in a crop is vital to understanding the potential for yield loss and economic risk. The identification of a particular virus might be difficult by visual inspection as different viruses can lead to the development of the same or similar disease symptoms. Historically, some viruses were known to infect carrot crops and had been reported in the UK in the past but not at a high prevalence, in part due to a lack of techniques for screening and lack of crop surveillance. The development of serological methods such as Enzyme-linked immunosorbent assay

(ELISA), or molecular methods such as Polymerase chain reaction (PCR), provide us with the opportunity to screen a large number of samples with a more rapid turnaround.

Internal necrosis in carrots had been seen sporadically in the UK for more than 10 years. Limited surveys carried out in 2011 also suggested a possible link between internal necrosis and virus infection in carrots (Fox, 2011). However, the majority of the samples were negative for PYFV and CMD when tested by reverse-transcriptase PCR (RT-PCR) raising the possibility that other viruses could be the agents responsible for the development of necrotic symptoms.

In 2013, symptomatic samples from a carrot field in North Yorkshire were examined and tested using RT-PCR for the presence of PYFV and CMD viruses. Results indicated no correlation between the presence of these viruses and the development of necrotic disease symptoms (Fox, 2013). A subsample of carrot roots with a range of symptoms were selected for further analysis using Next Generation Sequencing (NGS). The study found *Carrot yellow leaf virus* (CYLV) as the agent most strongly correlated with the development of the necrotic symptoms (Adams *et al*., 2014). This virus was known to affect the crop but it had not been previously detected infecting carrots in the UK. Of the symptomatic carrots, 98 % tested positive for CYLV, and the statistical analysis revealed that the removal of the virus from the carrot population would result in an estimated reduction of 96 % of necrosis. In the same study, additional previously unknown viruses were also found: a new Closterovirus tentatively named Carrot closterovirus 1 (CtCV1), similar to CYLV was found in the necrotic samples, two new Betaflexiviruses from the genus *Chordovirus*, *Carrot chordovirus 1* and *Carrot Chordovirus 2* (CtChV1 and CtChV2) and a new member of the *Torradovirus* genus, named *Carrot torrado virus1* (CaTV1) were also found, mainly in unaffected samples. The impact of these new viruses was not known and further studies would therefore be required.

Although the previous study was able to find a probable correlation between internal necrosis and infection with CYLV, it also revealed how little information is known about the actual virus communities present in carrot crops in the UK, their epidemiology and impact in the field. Surveys usually provide an important source of information to quantify the importance and epidemiology of one or a specific range of pathogens and to assist agronomists and plant breeders with crop production. In this study, surveys of different carrot fields were carried out in 2014 and 2015 across the main carrot growing areas of the UK and in 2016 in the Southwest of France to assess and establish the incidence of the new and known viruses. A limited survey of weeds including weed extracts stored by the Warwick Crop Centre (UK) was carried out under AHBD Horticulture funding (project FV382v) to look for possible reservoir hosts and study genome diversity of CYLV and CtCV1.

2.2 Material and methods

2.2.1 Field surveys

Carrot surveys across the UK and France were carried out during the years 2014-2016 (Table 1) in order to establish virus incidence and prevalence for the known and new carrot viruses.

Different carrot fields in North Yorkshire and Norfolk, known to be at risk of development of necrosis, were chosen in 2014 and 2015. In 2014 three field surveys were carried out in carrot crops in the region of North Yorkshire (on farms in Strensall, Sand Hutton and Sutton on the Forest). This area was later used as "control area" for a broader sampling in 2015, when three carrot fields were assessed in North Yorkshire and three more in Norfolk to ascertain if virus distribution was limited to a discrete geographic region in the UK. In 2015, a further field in North Yorkshire (Stamford Bridge) was assessed early and late in the season in order to look for possible differences between dates. In June 2015, samples of Cow Parsley (*Anthriscus sylvestris*) were collected from the field margins of the carrot crops in Stamford Bridge and Sutton on the Forest.

The sampling strategy in the UK was carried out marking grids of 100 x 100 m, collecting a sample every 10 m regardless of the symptomatic status of the plant at that point. A total of 121 samples were collected from each field. Nantes (Cv. Nairobi) is the carrot variety predominantly cultivated in the UK and all the work has been carried out with this variety.

Sampling in France was carried out in October 2016 in the region of Aquitaine, in the southwest of France, where 40-50 % of the carrot production of the country takes place. Samples (60 from each field) were randomly collected from each of the five fields from different planting dates. A total of 300 samples were taken and RNA was extracted for further testing. In these fields different carrot varieties were selected; Nantes (subtypes:

Bolero, Dordogne, Nantaise, Nerac, Purple haze, Romance, Texto, White Satine and Yellow Stone) and Imperator (SugarSnax) (Table 2.1).

Table 2.1: Details of samples collected during the 2014-2016 study, including details on location of the fields visited, planting and sampling dates and cultivars.

Field identifier	County	Variety	Date planted	Date sampled
UK				
Strensall	Yorkshire	Nantes	Unknown	06/08/2014
Sand Hutton	Yorkshire	Nantes	Unknown	07/08/2014
Sutton on the Forest	Yorkshire	Nantes	Unknown	21/08/2014
UK				
Stamford Bridge	Yorkshire	Nantes	21/02/2015	03/06/2015
Stamford bridge late	Yorkshire	Nantes	10/03/2015	27/08/2015
Sutton on the Forest	Yorkshire	Nantes	02/03/2015	03/07/2015
Elveden	Norfolk	Nantes	Unknown	28/07/2015
Hingham "high risk"	Norfolk	Nantes	Unknown	28/07/2015
Hingham "low risk"	Norfolk	Nantes	Unknown	28/07/2015
FRANCE				
CTIFL*	Aquitaine	Nantes	Jan – July	06/10/2016
Roumagne	Aquitaine	Nantes/ Imperator	28/05/2016	06/10/2016
Lanton	Aquitaine	Imperator	02/06/2016	25/10/2016
Parentis conventional	Aquitaine	Nantes	03/06/2016	25/10/2016
Parentis organic	Aquitaine	Nantes	20/06/2016	25/10/2016

*CTIFL: Centre Technique Interprofessionel des Fruits et Legumes.

2.2.2 Extraction of samples

All UK samples were extracted using the KingFisher® mL system (Thermo Labsystems) according to the protocol described in Mumford *et al*. (2002). Samples were eluted in 200 µl of molecular grade water and stored at -20 °C until further testing. Extraction of the French samples was carried out using the RNA isolation from plant kit (Macherey-Nagel) following the manufacturer´s instructions and elution was performed in 40 µl of RNase free water.

2.2.3 Quality assessment using the *Cytochrome oxidase* assay (COX)

The quality of all extracted samples was evaluated using a qPCR test designed to the *Cytochrome oxidase* gene using the following primers and probe: **COX F:** CGTCGCATTCCAGATTATCCA, **COX R:** CAACTACGGATATATAAGRRCCR RCCRRAACTG and **COX P:** VIC-AGGGCATTCCATCCAGCGTAAGCA-TAMRA (Weller *et al*., 2000).

2.2.4 Testing of samples using RT-qPCR

The viruses selected for the testing were the novel viruses found in the NGS study; CYLV, CaTV1 and CtCV1 and the already known and established viruses; CMoV, CtRLV and CtRLaVRNA. PYFV was only tested in samples collected in 2014. Primers and probes for all the viruses except CaTV1 were obtained from Adams *et al*. (2014) (Table 2.2). Primers and probes for CaTV1 RNA1 and CaTV1 RNA2 were designed using Primer Express 2.0 (Applied Biosystems) (Rozado-Aguirre *et al*., 2016). Probes were labelled with the reporter dye FAM (6-carboxyfluorescein) and the quencher dye TAMRA (tetra-methylcarboxyrhodamin).

Table 2.2: RT-qPCR primer sequences used for all the viruses tested.

Primer name	Sequence 5'-3'
CYLV F	AAG ATT CTC TTG TAA CGA AGG TTT CC
CYLV R	GCC GCC TCC ACG ATC AC
CYLV P	FAM-AGA CCT CAC TAT GCT AAA CCC GAG CCG G-TAMRA
CtCV1 F	GCC TCC CGC TTG TTG GA
CtCV1 R	AGC CGC CAA CGT CTA TGA AG
CtCV1 P	FAM-AAT AGG ACC GTC GCG AGT TTC TGC TCT-TAMRA
CaTV1 RNA1 F	CCG TTG TTA TTC GTC TTC CTC AA
CaTV1 RNA1 R	TGG ATG ATT GTA AAT ACT GCA CCA T
CaTV1 RNA1 P	FAM-TTC AGA GGT GTT TAC GTG AGA TCG GGA TG-TAMRA
CaTV1 RNA2 F	TTA CAA AGA CTA CTG GTG ATC GTG ACT T
CaTV1 RNA2 R	ATT CGT ACA AAC CCA CCT CAA AG
CaTV1 RNA2 P	FAM-AGA GTT GGA AAT GAT GCA ACC CAT GAT AGC-TAMRA
CtRLV F	CCC GGG TCT TGT TCC AGT CT
CtRLV R	GCC AAA WCC GGG TTT TGA R
CtRLV P	FAM-AGA ACA AGT TAG AAA TMG CGC TTT GGA GAG C-TAMRA
CtRLaVRNA F	CGK TTA GTT CGG ATT ACG ACA TAG TAT TT
CtRLaVRNA R	GTA AGT GAG CAC CCT GTT TAC CAA
CtRLaVRNA P	FAM- ATA CTA ACT CTA GGA ATA AYT TGA TGC GAG G-TAMRA
CMoV F	CCT ACA CGC CGC GAT GA
CMoV R	CRA CTG CGT GGA CTT TTC CG
CMoV P	FAM-AAC GAT ATG GGA ACC ACG ACC AGG G-TAMRA

***CYLV** (*Carrot yellow leaf virus*), **CtCV1** (Carrot closterovirus 1), **CaTV1** (*Carrot torrado virus 1*), **CtRLV** (*Carrot red leaf virus*), **CtRLaVRNA** (Carrot red leaf associated RNA), **CMOV** (*Carrot mottle virus*).

- **Testing in the UK**

The RT-qPCR setup was carried out using 384-well plates using an automated liquid handling robot (Star line, Hamilton). Samples were tested by adding 5 µl of extracted RNA to a reaction mix containing 15 µl of One–Step qRT-PCR Mastermix (Primer Design, UK) for CaTV1 RNA1 and RNA2 and iTaq^{TM} Universal One-Step RT-qPCR (Bio-Rad, US) for the remaining viruses. Before testing, extracts were diluted 1:5 in molecular grade

water. Assays with Primer Design mastermix were performed using the 7900 HT systems (Applied Biosystems) and cycling conditions were as follows: 10 min at 55 °C for reverse transcription, 8 min at 95 °C, and then 40 cycles of 10 sec at 95 °C and 1 min at 60 °C. Assays with the Bio-Rad mastermix were performed using either the 7900 HT, 7500 (Applied Biosystems) or Viia7 (Applied Biosystems). Cycling conditions consisted of 10 min at 50 °C, 2 min at 95 °C, followed by 40 cycles of 10 sec at 95 °C and 1 min at 60 °C. A concentration of 300 nM of Forward and Reverse primers and 100 nM of the Probe were used in all cases.

- **Testing in France**

RNA extracts were tested by adding 1 µl of the sample to each well in a 96-well plate containing 9 µl of One-Step RT-qPCR (Bio-Rad, US), 300 nM of Forward and Reverse primers and 100 nM of probe. Reactions were run on the Lightcycler® 480 system (Roche). Cycling conditions were the following: 10 min at 50 °C for initial reverse transcription, 2 min at 95 °C, followed by 40 cycles of 10 sec at 95 °C and 1 min at 60 °C.

2.2.5 Analysis of the results and use of positive controls

Results were analysed using the SDS 2.4 Software (Applied Biosystems) and analysis parameters (baseline and threshold) were set according to the manufacturer's instructions. A Ct value under 40 was considered as a positive result. For the testing carried out in France, results were analysed using the LightCycler® 480 SW 1.5 (Roche). Positive controls were field samples identified as being infected in studies from previous years.

2.2.6 NGS library preparation and setup

RNA extracts from Cow Parsley (*Anthriscus sylvestris*) and Hogweed (*Heracleum sphondylium*) samples collected in 2012 from a number of locations around the UK were prepared for NGS analysis. A total of 24 samples known to be positives using RT-qPCR for CYLV, CtCV1 or both were selected. cDNA synthesis and fragmentation was performed using ScriptSeq™ v2 RNA-Seq kit (Epicentre, Illumina) following manufacturer's instructions. Libraries were quantified using Qubit-HS double stranded DNA kits (Invitrogen, UK) and quality checked using a Tapestation (Agilent). Equimolar quantities of each sample were pooled in a single tube and purified using Ampure XP beads (Truseq manual). Pooled libraries were quality assessed and sent for MiSeq sequencing (Illumina) according to the manufacturer's instructions.

2.2.7 Sequencing data analysis

Data from the NGS run was subjected to an initial QC step and trimming to remove low quality reads. A phred quality score (Q score) of 20 was used in order to assess the accuracy of the sequencing. Transcripts were then assembled using Trinity, and BlastX was used to identity known sequences. Sequence alignments and comparisons were carried out using ClustalW and MEGA6.

2.3 Results

2.3.1 Symptoms in the field

The plant samples collected displayed a diverse range of typical virus-disease symptoms such as chlorosis, reddening, necrosis and stunting (Figure 2.1). In some of the fields visited, yellow patches with fewer plants were also observed. The presence of symptoms depended on the time of the season the sampling took place with plants sampled early having fewer symptoms than plants from later sampling dates.

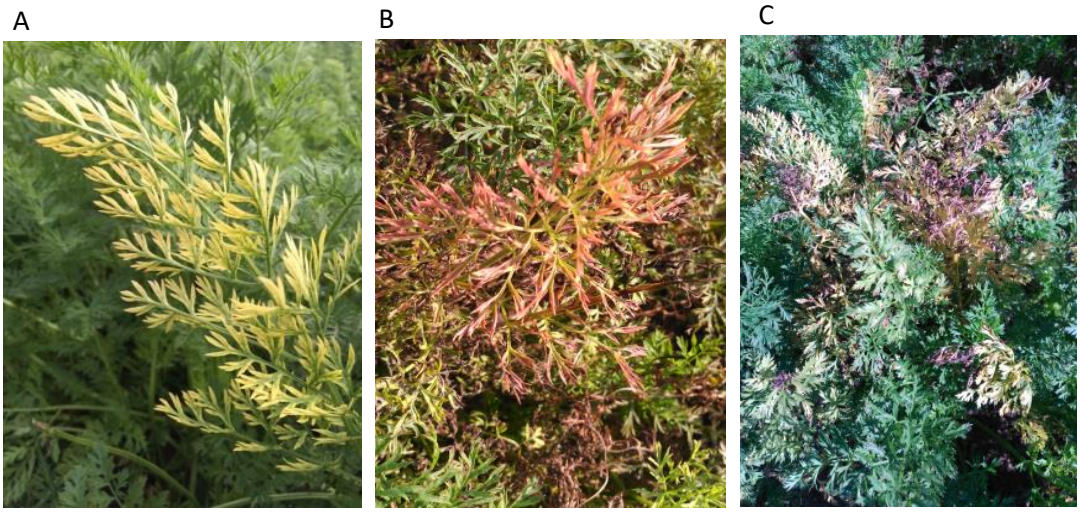


Figure 2.1: Viral symptoms found in the fields visited during the 2014-2016 period: (A) Yellow carrot leaves found in High Roans field in 2014. (B) Reddening in carrot leaves collected in High Roans in 2014. (C) Necrotic leaves collected in CTIFL (Centre Technique Interprofessionel des Fruits et Légumes) in France in 2016.

2.3.2 Field testing

2014 - UK

During 2014, three fields in North Yorkshire were sampled using the 100 m x 100 m grid pattern. Samples (363) were collected and tested for the presence of the known and novel carrot viruses. The incidence of infected plants found was 64.4 % and 67.7 % in Strensall and Sand Hutton respectively but this percentage dropped to 33.3 % in Sutton on the Forest. The prevalence of each virus from each plot was as follows; the percentage of plants infected was 26.7 %, 13.3 % and 10.0 % for CaTV1 in Strensall, Sand Hutton and Sutton on the Forest respectively (Figure 2.2a). Incidence levels of 25.1 % and 33.3 % for CYLV were found in Strensall and Sand Hutton respectively, and a lower percentage (5.4 %) in Sutton on the Forest. Prevalence of CtCV1 was also found to be higher in Strensall (21.5 %) and Sand Hutton (37.4 %) and a prevalence of 5.8 % was observed in Sutton on the Forest. Such differences in percentages of infection between fields were not found when samples were tested for the viruses associated with CMD: 28.6 %, 34.4 % and 18.5 % in Strensall, Sand Hutton and Sutton on the Forest respectively. Conversely, PYFV was absent in all the fields except from Sand Hutton where 0.9 % of the plants were infected.

2015 - UK

During the 2015 season, three fields in North Yorkshire were sampled following the same 100 x 100 m sampling pattern. On the advice of local growers and agronomists, some carrot fields thought to be at high and low risk of infection were identified and selected. Two closely located fields in Stamford Bridge (Yorkshire) were sampled early and late in the season to look at possible differences between dates. Additionally, three further fields in Norfolk were sampled to assess the geographical distribution of the novel viruses in the UK. Along with Yorkshire and Lancashire, this area is known to be one of the main regions of carrot production in the UK. Results indicated some marked differences with those obtained in 2014 in Yorkshire (Figure 2.2b). The viruses of the CMD complex were found to be present at greater incidence in late season sampling of the field in Stamford Bridge and the fields in Norfolk where the infection level reached 100 % in samples from Hingham "high risk", indicating infection in every plant sampled in that field. The closteroviruses, CYLV and CtCV1, were present with incidences of 38 % and 20.7 % respectively, in the late sampled field in Yorkshire. Both viruses were found at lower prevalence in the early season sampling in Sutton on the Forest (0.8 % CYLV and CtCV1) and Stamford Bridge (2.5 % CtCV1). CaTV1 was absent from all the fields in that region.

The prevalence of the novel viruses in each field sampled in Norfolk were as follows: CaTV1 was found to be present with incidence of infected plants up to 39.5 %, 64.5 % and 7.4 % in Elveden, Hingham "high risk" and Hingham "low risk" respectively. CYLV was present in Norfolk with an incidence of 31.4 %, 43 % and 14 % and CtCV1 with an incidence of 91 %, 14.9 % and 10.9 % in Elveden, Hingham "high risk" and Hingham "low risk" respectively.

2016 - France

In October 2016, field surveys were carried out in the region of Aquitaine, in the southwest of France. Symptomatic and symptomless samples were randomly selected from each field and a total of 300 samples were collected in the region. The fields located in Lanton and one from Parentis were subjected to organic farming practices whilst the remaining sites were subject to conventional farming. CaTV1 was found with an incidence of 26.7 % at CTIFL with year-round carrot cultivation and no vector control (Figure 2.2c). However, no CaTV1 was found in any of the other fields sampled. CYLV and CtCV1 were absent from all samples collected in all the fields. The viruses associated with CMD were found in all the fields at different incidences: 83.3 % in CTIFL, 23.4 % in Roumagne, 9.3 % in Lanton and 31.2 % and 11.3 % in Parentis conventional and organic respectively.

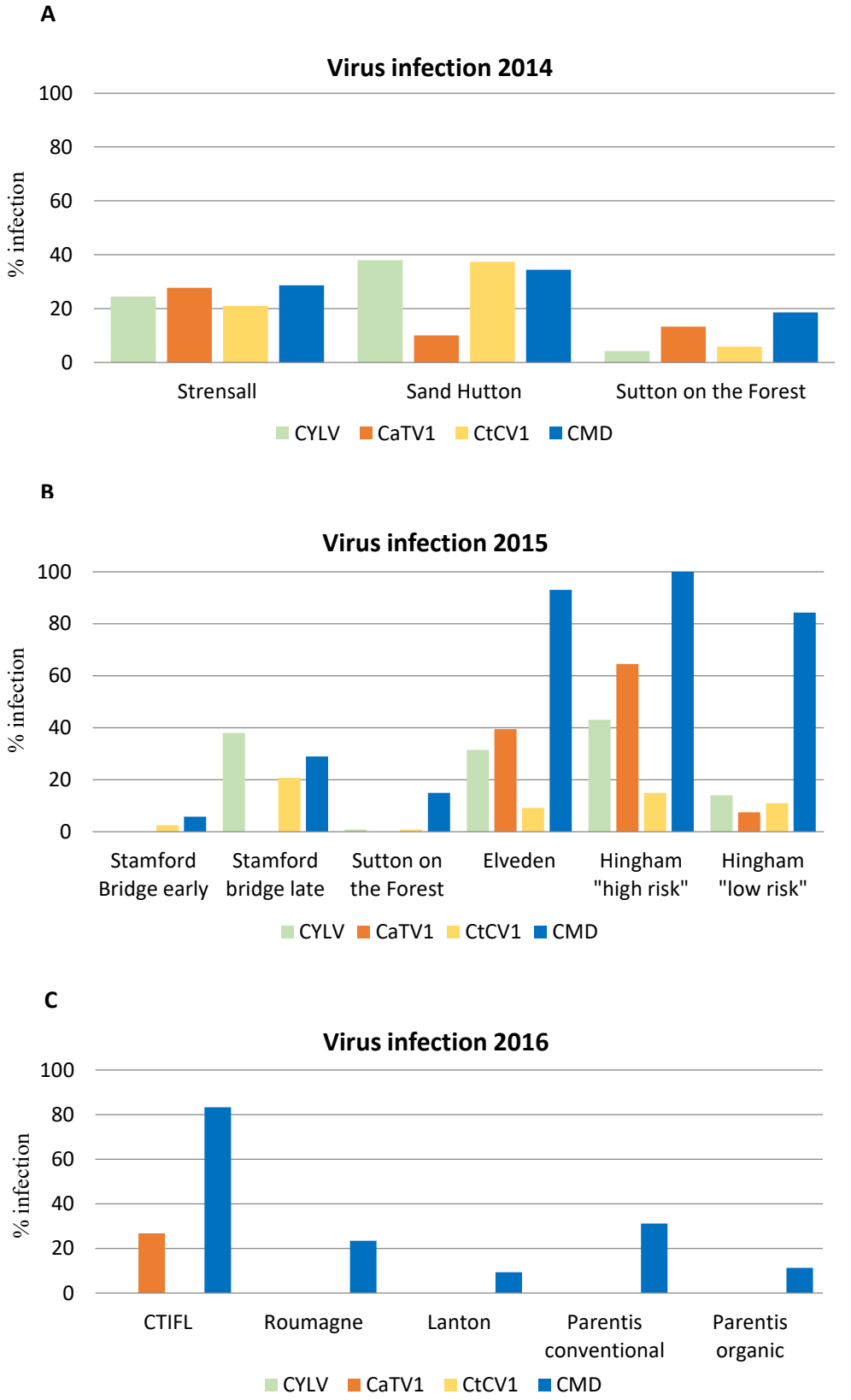


Figure 2.2: Percentages of plants infected with each virus obtained from field testing in UK (A), 2015 in the UK (B) and 2016 in France (C). Notice that infection with one of of the complex was considered as infection with the complex

2.3.3 Weed samples

In 2015, Cow Parsley samples surrounding carrot fields were sampled in order to look at possible virus reservoirs. A total of 26 samples, 16 from Stamford Bridge and 10 from Sutton on the Forest, were collected and tested for all the viruses as previously described: CtCV1 and CYLV were the most prevalent viruses in Stamford Bridge and Sutton on the Forest, where infection levels of 68.8 % and 70.0 % were found respectively (Table 2.3). CtRLV was present with an incidence of 18.8 % and 40.0 % in Stamford Bridge and Sutton on the Forest respectively. No CaTV1 was detected in any of the samples tested.

RNA extracts (938) from Hogweed, Cow Parsley, Hemlock (*Conium maculatum*) and Rough Chervil (*Chaerophyllum temulum),* Alexander's (*Symyrnium olusatrum*) and Ground elder (*Aegopodium podagraria*) were collected in 2011 and 2012 all around the UK by the Warwick Crop Centre and tested for the viruses associated with CMD and PYFV. The same samples were tested for the presence of CaTV1, CYLV and CtCV1 by the Virology group in FERA in 2016. In 2011 samples, 144 out of 478, tested positive for CtCV1, with Cow Parsley being the most predominant host (111) followed by Hogweed (Table 2.4). CYLV tested positive in 41 and 17 samples in Cow Parsley and Hogweed respectively. CtCV1 was again the most prevalent virus in 2012, when 460 samples were tested, and 111 tested positive in Cow Parsley (97) and Hogweed (14). CYLV was also found in Cow Parsley (23) and Hogweed (2). No CaTV1 was found in any of the extracts tested from both years.

Table 2.3: Results from the weed testing carried out in North Yorkshire in 2015 using qPCR.

Field	% of plants infected					
	CtRLV	**CMoV**	**CRLaVRNA**	**CYLV**	**CtCV1**	**CaTV1**
Stamford Bridge	18.8	0	0	68.8	37.5	0
Sutton on the Forest	40	0	0	20	70	0

CYLV (*Carrot yellow leaf virus*), **CtCV1** (Carrot closterovirus 1), **CaTV1** (*Carrot torrado virus 1*), **CtRLV** (*Carrot red leaf virus*), **CtRLaVRNA** (Carrot red leaf associated RNA), **CMoV** (*Carrot mottle virus*).

Table 2.4: Results from the testing of Warwick weed samples carried out by the Virology team at FERA using qPCR.

Species	2011			2012		
	CYLV	**CtCV1**	**CaTV1**	**CYLV**	**CtCV1**	**CaTV1**
Alexander's		4			1	
Cow Parsley	41	111		23	97	
Ground Elder	2	2			0	
Hemlock	1	1				
Hogweed	17	24		2	14	
Rough Chervil		2				
Total	61	144	0	25	112	0

CYLV (*Carrot yellow leaf virus*), **CtCV1** (Carrot closterovirus 1), **CaTV1** (*Carrot torrado virus 1*), **CtRLV** (*Carrot red leaf virus*), **CtRLaVRNA** (Carrot red leaf associated RNA), **CMoV** (*Carrot mottle virus*).

2.3.4 Genome diversity

RT-qPCR positive samples for CYLV (24) and CtCV1 (13) from the 2012 Warwick Crop Centre samples were selected in order to study genome diversity of both viruses. The new viruses from the genus *Chordovirus*, *Carrot chordovirus 1* (CtChV-1) and *Carrot chordovirus 2* (CtChV-2), identified in the previous NGS study (Adams *et al*., 2014), were found to be present in samples from Hogweed and Cow Parlsey. Comparisons of the sequence fragments obtained with carrot sequences from 2013 (A.C. KF533710 and KF533711), revealed levels of nucleotide identity ranging from 77 to 90 %. CYLV sequences resulting from the analysis of four samples revealed levels of identity of 98-99 % when compared with the published sequence from carrot (KF533708). CtCV1 sequences were also compared to the published sequence from carrot (KF533697) with nucleotide identity ranging between 89-99 %. However, assembly of the virus sequences from the NGS run resulted in genome sequences of only 200-300 bp for both CYLV and CtCV1 and no further comparisons could be made. NGS results also suggested the presence of PYFV (NC_003628), CtRLV (AY695933), *Carrot cryptic virus*, CaCV (FJ550605), *Rice tungro spherical virus*, RTSV (M95497) and Citrullus lanatus cryptic virus, CiLCV (KY08128).

2.4 Discussion

Field surveys were carried out around the UK and France in the period 2014-2016 in order to assess the presence of known and novel carrot viruses. The first results from 2014 in Yorkshire indicated a marked difference in virus incidence between fields, with a lower percentage of infection in Sutton on the Forest compared to the other two fields sampled (Strensall and Sand Hutton). All the fields were from the same grower and subjected to the same agronomic practices. The most significant difference between the fields was a later planting date at Sutton on the Forest. A later planting date would potentially reduce the time the crop was exposed to virus vectors as well as the time at which more susceptible seedlings are exposed in the field, which might explain the differences in virus incidence. High incidence of the novel and other viruses which had been previously overlooked, such as CYLV and CtCV1, was found in all fields. Surveys in 2015 were carried out in a broader geographic area and results indicated higher levels of infection in the fields in Norfolk (Elveden, Hingham "high risk" and Hingham "low risk") than in the ones sampled in Yorkshire (Stamford Bridge and Sutton on the Forest). Transmission vectors such as aphids might play a key role in these differences. These tend to appear earlier and in greater abundance in the south of the country, so viruses could be more easily transmitted to the crops and for longer periods. Again, greater virus incidence was found in the late sampled field in Stamford Bridge where the crop was exposed to the viruses and their vectors for a longer period of time. Results from France indicated the first report of CaTV1 in that country and outside the UK (Rozado-Aguirre *et al*., 2017). However, no CYLV or CtCV1, were found in any of the samples.

The collection of epidemiological data is an essential requirement in order to assess the presence of a new pathogen within a crop. As CaTV1 and CtCV1 were first identified by sequencing approaches, further information on the epidemiology, transmission and impact was required to make decisions about the plant health impact. CaTV1 was found to

be present in 2014 and 2015 in different areas across the UK and it was also identified in field surveys carried out in France in 2016. However, due to the presence of mixed infections, no relation between the presence of this virus and the development of disease symptoms could be established. CtCV1 was not found in France but the high incidence of this virus in the UK and its similarity to CYLV might suggest that it is also linked to the induction of internal necrosis symptoms in a similar way to CYLV, although further studies are needed to confirm this hypothesis.

In order to establish control strategies, it is necessary to define which susceptible species are the most likely virus reservoirs under natural conditions. Weeds surrounding crop fields are often postulated as the main source as they play an important role in plant viral diseases, harbouring many species which are later transmitted to the field (Duffus, 1971, Wisler *et al*., 2005). They also compete with the crop for light, water, nutrients, and physical space within the field. Transmission studies with CYLV from weeds to carrots, *Nicotiana benthamiana* and chervil (*Anthriscus cerefolium*) using four different aphid species (*Myzus persicae, Cavariella aegopodii, C. pastinaceae* and *C. theobaldi*) led to very low infection rate or no transmission (Fox *et al*., 2015). Limited weed surveys from carrot field margins in 2015 and testing carried out by the Virology group at FERA indicated high levels of infection for CYLV and CtCV1 in the samples, with Cow Parsley (*Anthriscus sylvestris*) being the predominant host. These results raised the possibility that both viruses could be transmitted from weeds to carrots by the corresponding vector. CaTV1 has not been found in any of the weed extracts tested so far, indicating that infection could come directly from carrots or that there is still an unknown alternate host. Another possibility is that CaTV1 is circulating in carrot crops, since in the UK, continuous cropping is employed where the plants are stored in the field under straw. Consequently, the previous year´s crop might be a source of infection for the forthcoming season. This hypothesis was previously proposed when alternate hosts and transmission of the viruses associated with CMD were investigated. Genome diversity studies using CtRLV sequences from weeds and

carrots indicated that sequences from both hosts clustered in different clades, suggesting that infection in carrots could not come from the infected weeds surrounding the field (unpublished data). Results also raised the possibility that infection could come directly from carrots under straw from the previous year. Infection with CaTV1 could take place in the same way as the viruses associated with CMD. It was noticeable that from the study conducted in France, this virus was only found in a single field characterized by year-around carrot cultivation and not in the other crops studied. Preliminary studies with seeds also suggested CaTV1 could be seed transmissible but further investigations are needed to confirm this observation (Adrian Fox, personal communication).

No clear results and conclusions could be reached from the population diversity studies performed with CYLV and CtCV1. Low quality sequences with short reads were obtained, probably due to the quality of the samples, which had been collected in 2012. The extraction method used is unknown, and this can also affect the results obtained from the sequencing. In spite of the low quality, sequences from the new Chordoviruses were identified in Cow Parsley and Hogweed, indicating for the first time the presence of both viruses in weed samples. Besides, CYLV and CtCV1 sequences obtained from the sequencing indicated high levels of identity when compared with the sequence from carrot, suggesting that both viruses may be transmissible from those weed species to the crop. However, results were not conclusive and the method should be repeated with fresher samples to confirm this hypothesis.

The collection of epidemiological data is also the key for the development of effective disease management strategies (Jones, 2004). For instance, several control measures have been taken in the past to control the spread of PYFV and CMD viruses. Good control has occasionally been achieved against CMD by planting new fields at a substantial distance from overwintered fields ensuring safe planting regions, or applying

insecticides against the vectors. A new management strategy was set in 2011 for the control of PYFV which included the set-up of field water traps in the crops. These should be assessed weekly to ascertain the presence of the willow carrot aphid and establish the starting date of the annual migration into the crop (Morgan *et al*., 2004). Growers should also begin their spray programme once the aphids had been trapped. Avoiding seedling emergence during peak aphid populations or sowing when the vector is at low activity level have also been shown to be beneficial measures to manage *Carrot virus Y* in Australia (Latham and Jones, 2004). Looking at previous results from the field surveys, avoiding continuous, all-year-round cropping might also be a beneficial preventive measure in order to avoid CaTV1 virus infection. Most of the control strategies have been focused on the control of the willow-carrot aphid as this vector is responsible for the transmission of PYFV and CMD. However, CaTV1 has been seen to be effectively transmissible by the aphid species, *Myzus persicae* (Rozado-Aguirre *et al*., 2016), indicating a new potential vector which might play a key role in carrot virus epidemics. Further studies regarding how the new viruses are transmitted to the crop would be recommended in the future in order to control the incidence and aid development of effective control strategies.

2.5 First report of *Carrot torrado virus1* (CaTV1), a member of the *Torradovirus* genus, on carrots in France

Rozado-Aguirre, Z., Marais, A., Svanella-Dumas, L, Faure, C., Latour, F., Villeneuve, F., Dickinson, M., Fox, A., Boonham, N., Candresse, T. (2017). First report of Carrot torrado virus1 (CaTV1), a member of the Torradovirus genus, infecting carrots in France, Plant Disease, Vol 101, Number 7.

In 2013, a novel virus named *Carrot torrado virus 1* (CaTV1) was identified in the UK using Next Generation Sequencing (NGS) in asymptomatic carrots (*Daucus carota*) during an investigation of the causes of root internal necrosis (Adams *et al*., 2014). It was recognised as a new member of the genus *Torradovirus*, an emerging group of viruses in the family *Secoviridae* affecting a range of crops worldwide.

Epidemiological studies carried out in the UK in 2014 and 2015 indicated levels of infection up to 26.7 % and 64.5 % respectively. Experiments also suggested that CaTV1 may be transmitted by the aphid species *Myzus persicae* and *Cavariella aegopodii* (Rozado-Aguirre *et al*., 2016). So far, CaTV1 has only been described in the UK and its potential presence elsewhere in the world remains to be investigated. In October 2016, a total of 312 asymptomatic and symptomatic leaf samples presenting reddening, chlorosis and necrosis were collected from several carrot fields in Aquitaine, in the Southwest of France. Total RNAs were extracted using the Nucleospin® RNA plant kit (Macherey-Nagel) and eluted in 40 µl of RNase-free water. Sample extracts were tested using a qPCR designed to amplify the *Cytochrome oxidase* (COX) mRNA, used as a measure of the quality of the extraction, and subsequently used as template for RT-qPCR assays for the detection of CaTV1 RNA1 and RNA2 using two pairs of specific primers (Rozado-Aguirre

et al., 2016). Samples were also tested for a range of known viruses affecting carrots (Carrot mottle dwarf disease viruses: *Carrot red leaf virus*, Carrot red leaf associated virus and *Carrot mottle virus).*

The results obtained showed a CaTV1 infection level of 26.7 % (16 out of 60 samples) in one of the sampled fields, which was characterized by year-around carrot cultivation and an excessive presence of wild Apiaceae. CaTV1 was not detected in the four other locations sampled. To confirm these results, four of the CaTV1 positive samples were tested by two-step RT-PCR assays using *Torradovirus* generic primers specific for each of the genomic RNAs (Verbeek *et al*., 2012). After gel electrophoresis, RT-PCR products of 370 bp (RNA1) and 514 bp (RNA2) were obtained for each of the samples. The identity of CaTV1 was further established by the direct sequencing of representative RT-PCR amplicons (accession numbers KY636379 and KY636380) finally confirming the presence of CaTV1 in the RT-qPCR positive samples. Comparison of the sequences obtained with the CaTV1 UK sequences (KF533719 and KF533720) indicated 81 % and 97.4 % of nucleotide (nt) and amino acid (aa) identity respectively in the amplified partial polymerase fragment (RNA1) and 85 % (nt) and 48.7 % (aa) identity respectively in the partial coat protein region (RNA2), demonstrating that the French CaTV1 isolate analysed is significantly different from the UK isolate.

The evaluation of a possible contribution of CaTV1 to symptom development in France was not possible because of the simultaneous presence in every CaTV1 positive plant of mixed infection involving agents of carrot mottle dwarf disease. This is the first report of the virus in France and outside the UK. Further studies are needed in order to establish the prevalence, transmission route and impact of this virus in the field.

References

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Chapter 3

Investigating the carrot virome using next generation sequencing (NGS)

In this chapter, carrot leaf samples from the UK and France were subjected to a Next Generation Sequencing study in order to study viral communities in these populations and identify new viral sequences present within the crop.

The PhD candidate collected and extracted all the selected samples. She performed library preparation and quality assessment of the extracts and prepared the samples for Next Generation Sequencing. She also helped in the analysis of the obtained sequences after quality checking and *de novo* sequence assembly of the reads, which was led by Dr. Ian Adams.

Abstract

Field samples collected in the UK and France were selected for a Next Generation Sequencing (NGS) study to look at the viral communities and identify new virus species that might be present in carrot crops. Results from France confirmed the presence of *Carrot torrado virus1* (CaTV1) in a single field and identified *Broad bean wilt virus* (BBWV) and *Carrot cryptic virus* (CaCV) as virus species present in the crop. Sequencing from the samples from the UK identified a new sequence of 5405 bp with 51 % aminoacid identity to *Poplar mosaic virus* (PopMV). Comparison with a similar but longer sequence of 8833 bp from Cow Parsley (*Anthriscus sylvestris*) indicated 89.1 % nucleotide identity between both sequences. Analysis within the coat protein region of the Cow Parsley isolate revealed 82.4 % nucleotide identity with Carrot Carlavirus, isolated from carrot in Germany in 2008. Results also indicated the presence of *Carrot necrotic dieback virus* (CNDV) in carrots in the UK.

3.1 Introduction

Following the development of molecular methods such as Enzyme-linked immunosorbent assay (ELISA), Polymerase chain reaction (PCR), or microarrays, Next Generation Sequencing (NGS) is quickly emerging as the diagnostic method of choice for the discovery and early detection of pathogens in relatively shorts periods of time. This technique has created a revolution in the field in the last years and it is currently used for discovery of new viruses, study of genome diversity, virus characterization, investigation of microbial communities and gene-expression profiling (Al Rwahnih *et al*., 2009; Ansorge, 2009; Barzon *et al*., 2011; Radford *et al*., 2012; Minutillo *et al*., 2015; Shendure *et al*., 2008). Traditional methods, such as ELISA or PCR lack the capacity to detect highly variable or poorly characterized pathogens. Conversely, NGS can also detect many more minority mutations and variants of a virus (Kutnaj *et al*., 2014). For instance, NGS was performed to characterize low prevalence nucleotide reverse-transcriptase inhibitor (NRTI) mutations in hepatitis virus B (Margeridon-Thermet *et al.*, 2009). This is particularly important in the face of new outbreaks of a disease, where the viruses need to be rapidly characterized. NGS has also played an important role in the detection of new pathogens which were not known to be present in imported samples. In 2014 in France, sequencing technologies were used to detect the plant virus *Sugarcane white streak virus* (SWSV) in plants originated from Egypt (Candresse *et al*., 2014). This virus had not been previously detected using routine quarantine detection methods. A new luteovirus, tentatively named Nectarine stem pitting-associated virus (NSPaV), was found in imported nectarine trees in the US, (Beg *et al*., 2015) and the discovery of a PCR non-detectable strain of *Plum bark necrotic and stem pitting associated virus* (PBNSPaV) led to the development of a PCR assay with a wider and more specific detection range (Marais *et al*., 2014)

Numerous assays using NGS have been carried out in order to study the virome of a particular host. For example, in Africa, viral infections are considered a major limitation

in sweet potato (*Ipomoea batatas*) production and a wide range of field-grown samples were collected to efficiently identify known and novel viral sequences present in the crop using NGS (Gutierrez *et al*., 2012). Information can then be used to gain a better knowledge on the virus distribution in a particular region, to predict possible threats and to establish preventive phytosanitary measures in order to avoid epidemics.

Since the introduction of the first platform in the mid 2000s, different NGS technologies have been developed and become commercially available. The first next generation platform to achieve commercial introduction in 2004 was the 454 FLX pyrosequencing platform, which was developed by 454 Life Sciences (Roche). Following 454 FLX, Illumina released their first Genome Analyzer in 2007 (Solexa GA) and more recently, new platforms such as SOLiD (Applied Biosystems), Ion Torrent (Life technologies) MinION (Oxford Nanopore) and a real-time sequencing technology by Pacific Bioscience have been released. MinION is not fully commercially available yet but it consists of a smaller device which can be plugged into a laptop. These technologies require similar sample preparation steps but they all utilize quite diverse biochemistries and sequencing protocols. Roche 454 FLX and Illumina have been widely used for years for the study of viral populations, discovery of novel viruses and complete genome sequencing and quasispecies analysis (Giampetruzzia *et al*., 2011; Li *et al*., 2012; Fabre *et al*., 2012). The choice of one sequencing platform over another usually depends on the starting material, the final objective of the study and the cost of the sequencing. The Illumina platform is currently leading the market of NGS platforms due to its reasonable price and accessibility.

The advent of NGS has been accompanied with the development of new bioinformatics tools in order to analyse and manage the huge amount of data generated from the sequencing (Blawid *et al*., 2016). These methods include analysis of sequence quality and *de novo* assembly of the obtained sequences. Aligned sequences are then

compared to genome sequences available in public databases. All the information generated from this approach needs to be carefully analysed and interpreted as it could have wide-ranging impact on all areas of virology, from diagnosis to pathogenesis, characterization and viral evolution, and virus management.

In this study carrot populations collected in the UK (2015) and in France (2016) were subjected to a NGS study to look at viral communities and potential pathogens established in each region.

3.2 Material and methods

3.2.1 Selection of samples

The samples used for this study were field samples collected in different regions across the UK in 2015 and in France in 2016. A total of 117 and 174 samples were selected from each country respectively. All of them had been tested previously using RT-qPCR for the following carrot viruses (Chapter 2): *Carrot yellow leaf virus* (CYLV), Carrot closterovirus 1 (CtCV1), *Carrot torrado virus1* (CaTV1), *Carrot red leaf virus* (CtRLV), Carrot red leaf associated RNA (CtRLaVRNA) and *Carrot mottle virus* (CMoV).

3.2.2 Extraction of the samples

Tables 3.1 indicates the number of extracts used for each NGS sample and their corresponding location. Plant material (200 mg) was ground in 10 volumes of CTAB grinding buffer (100 mM Tris-HCl pH 8, 2 % CTAB, 20 mM EDTA, 1.4 M NaCl, 1 % Na sulphite, 2 % PVP-40) and the ground solution was incubated 30 min at 65 °C. Samples were centrifuged at maximum speed for 5 min and the supernatant was treated with chloroform: isopropanol for nucleic acid separation. The upper phase was transferred to a clean tube and incubated at -20 °C overnight for precipitation with 5 M NaCl and ice iso-propanol. After 10 min centrifugation at maximum speed, the pellet was resuspended in TE buffer containing 1 % SDS, 5 M NaCL and ice-cold iso-propanol and the solution was incubated for 30 min at -20 °C. Finally, samples were centrifuged at maximum speed and pellet was washed by adding 70 % ethanol and the sample was centrifuged for 4 min. The dried pellet was resuspended in 100 µl of molecular grade water and stored at -80 °C until further testing.

Table 3.1: Samples selected for the NGS (Next Generation Sequencing) run collected in France in 2016 and in the UK in 2015.

France			UK		
Sample ID	**Extracts**	**Location**	**Sample ID**	**Extracts**	**Location**
2-1	1-8	CTIFL*	*3-1*	H1-H4	Stamford Bridge
2-2	9-16	CTIFL	*3-2*	1-5	Stamford Bridge
2-3	17-24	CTIFL	*3-3*	6-10	Stamford Bridge
2-4	25-32	CTIFL	*3-4*	41-45	Stamford Bridge
2-5	33-40	CTIFL	*3-5*	46-50	Stamford Bridge
2-6	41-48	CTIFL	*3-6*	201-205	Sutton on the Forest
2-7	49-55	CTIFL	*3-7*	206-210	Sutton on the Forest
2-8	56-62	CTIFL	*3-8*	211-215	Sutton on the Forest
2-9	63-69	Roumagne	*3-9*	216-220	Sutton on the Forest
2-10	70-76	Roumagne	*3-10*	291-295	Elveden
2-11	77-83	Roumagne	*3-11*	296-300	Elveden
2-12	84-90	Roumagne	*3-12*	321-325	Elveden
2-13	107-113	Roumagne	*3-13*	326-330	Elveden
2-14	114-120	Roumagne	*3-14*	351-354	Elveden
2-15	121-127	Roumagne	*3-15*	391-395	Hingham “high risk”
2-16	128-134	Lanton	*3-16*	396-400	Hingham “high risk
2-17	135-141	Lanton	*3-17*	431-435	Hingham “high risk
2-18	201-207	Parentis	*3-18*	436-440	Hingham “high risk
2-19	208-214	Parentis	*3-19*	481-484	Hingham “high risk
2-20	215-221	Parentis	*3-20*	531-535	Hingham “low risk”
2-21	222-228	Parentis	*3-21*	536-540	Hingham “low risk”
2-22	229-235	Parentis	*3-22*	561-565	Hingham “low risk”
2-23	236-242	Parentis	*3-23*	591-595	Hingham “low risk”
2-24	243-250	Parentis	*3-24*	596-600	Hingham “low risk”

***CTIFL**: Centre Technique Interprofessionel des Fruits et Légumes.

3.2.3 Preparation of the library

RNA bulked extracts for NGS were quantified using the Qubit-RNA HS Assay kit. Ribosomal RNA depletion and library preparation was performed using the Ribo-Zero™ rRNA Removal Reagents kit (Illumina) and ScriptSeq™ v2 RNA-Seq kit (Epicentre, Illumina) respectively following manufacturer’s instructions. Libraries were quantified using Qubit-HS double stranded DNA kit (Invitrogen, UK) and quality checked using a

Tapestation (Agilent). Equimolar quantities of each sample were pooled together in a single tube and subsequently purified using Ampure XP beads (Truseq manual). The purified sample was quantified and quality checked using Qubit-HS double stranded DNA kit and Tapestation. The samples were run on the MiSeq (Illumina) using MiSeq® Reagent Kit v3 600 cycles (Box 1 & 2) following manufacturer's instructions.

3.2.4 *De novo* sequence assembly and analysis of results

Sequenced data was subjected to an initial quality checking step setting a Phred quality score of 20 (Q20). Trinity software was used to assemble the transcripts and BlastX was used to identify known sequences from Genbank. Analysis and alignment of the sequences were carried out using MEGAN and MEGA6 respectively. Normalization of the reads were performed by calculation of the reads per kilobase per million mapped reads (RPKM). Results are shown as percentages of the RPKM for each sample and virus comparing to the sample with the highest RPKM value (100 %).

3.3 Results

3.3.1 Samples from France

Results from the sequencing of the samples from France are indicated in Table 3.2 where the longest sequences obtained for each virus are represented. Percentages of infection are represented using a colour scale, in which 0 % indicates the total absence of the viral sequence in that sample. CTIFL was seen to be the location with the presence of the most viruses compared to the other locations.

NGS of samples from France revealed the presence of *Carrot torrado virus1* (CaTV1) in samples collected in CTIFL, although complete sequences were not recovered. Pairwise comparisons of the obtained CaTV1 sequences with the sequence from Genbank indicated levels of nucleotide identity up to 95 % for RNA1 (KF533720.2) and 98 % for RNA2 (KF533719.2). Sequences corresponding to *Broad bean wilt virus* (AB084451), from the genus *Fabavirus*, were also obtained from sample 2-6. Small sequence fragments corresponding to the RdRp and coat protein region of *Carrot cryptic virus* (CaCV) (FJ550604 and FJ550605) were also identified. CtRLV, CRLaVRNA and CMoV, were also identified in samples from all the fields. No closteroviruses were found in any of the samples.

Table 3.2: Sequencing results from France are represented as percentages of RPKM (Reads per Kilobase per million mapped reads). A percentage of 100 % indicates the presence of the whole viral sequence in that sample. RPKM values for other samples are then represented as percentages of the presence of the viral sequence compared to the whole sequence (100%).

		CTIFL								Roumagne							Lanton		Parentis							
Virus	**Size (bp)**	**S1**	**S2**	**S3**	**S4**	**S5**	**S6**	**S7**	**S8**	**S9**	**S10**	**S11**	**S12**	**S13**	**S14**	**S15**	**S16**	**S17**	**S18**	**S19**	**S20**	**S21**	**S22**	**S23**	**S24**	**Pos**
CMoV	805	28%	13%	56%	0%	8%	100%	9%	0%	0%	0%	0%	0%	0%	0%	3%	0%	0%	0%	4%	25%	6%	22%	27%	4%	0%
CMoV	487	38%	13%	63%	4%	23%	100%	9%	0%	4%	0%	2%	17%	9%	0%	2%	0%	0%	0%	2%	33%	5%	14%	24%	0%	0%
CtRLaVRNA	709	100%	26%	19%	4%	1%	99%	3%	0%	3%	0%	0%	2%	7%	0%	0%	1%	0%	0%	69%	10%	12%	13%	7%	33%	0%
CtRLV	918	65%	19%	45%	31%	17%	28%	0%	0%	0%	0%	4%	30%	31%	0%	3%	0%	3%	0%	30%	100%	7%	17%	25%	0%	0%
CtRLV	874	8%	40%	100%	7%	0%	64%	0%	0%	0%	0%	4%	9%	7%	0%	3%	0%	0%	0%	19%	27%	6%	0%	22%	7%	0%
CaTV1	1470	100%	23%	25%	0%	2%	12%	0%	0%	0%	0%	0%	3%	2%	0%	0%	1%	0%	0%	2%	4%	0%	0%	0%	2%	0%
CaTV1	1525	100%	27%	69%	3%	0%	48%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	3%	0%	3%	0%	0%	0%
BBWV1	1029	0%	0%	0%	0%	0%	100%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
BBWV1	909	0%	0%	0%	5%	0%	100%	3%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	3%	0%	0%	3%	0%	3%	0%
CaCV	395	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	57%	100%	0%	0%	0%	0%
CaCV	326	0%	0%	0%	0%	0%	68%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	100%	0%	0%	0%	0%

***CMoV**: *Carrot mottle virus*; **CRLaVRNA**: Carrot red lead associated RNA**; CtRLV**: *Carrot red leaf virus*; **CaTV1**: *Carrot torrado virus 1*; **BBWV1**: *Broad bean wilt virus*; **CaCV**: *Carrot cryptic virus*.

3.3.2 Samples from the UK

Results from the sequencing are shown in Table 3.3. The longest sequences for each virus were selected and a cut-off of 1000 bp length was established for further analysis. Sequencing samples 1-9 from Yorkshire (Stamford Bridge and Sutton on the Forest) resulted in fewer virus sequences compared to samples from Norfolk (Elveden, Hingham "high risk" and Hingham "low risk") where viruses were found to be more abundant.

Results from the sequencing identified a new sequence of 5405 nucleotides in sample 3-15 (Hingham "high risk"). BlastX revealed the closest virus was *Poplar mosaic virus* ATCC PV257 (PopMV) (X65102.2) with 51 % aminoacid identity and identified motifs corresponding to a methyltransferase, helicase and a *Carlavirus* endopeptidase. Sequence alignment with a sequence of 8833 bp which had been been previously isolated from Cow Parsley (*Anthriscus sylvestris*) and had 54 % aminoacid identity to PopMV, indicated 89.1 % nucleotide identity between both sequences. Motifs corresponding to the RNA-dependent RNA polymerase (RdRp), viral methyltransferase, Carlavirus endopeptidase, movement protein (MP), helicase and viral coat protein were found in the sequence from Cow Parsley along with a PolyA tail. Pairwise comparisons indicated 70.3 % nucleotide identity to PopMV in the RdRp region and 82.4 % identity to Carrot Carlavirus in the coat protein region (EU881919).

Complete genomes of CYLV were recovered from samples from Hingham "high risk" and Hingham "low risk". These sequences have 95 % nucleotide identity to the complete CYLV genome sequence (FJ869862) and 99 % identity to the CYLV UK isolates (KF533705 and KF533708). Extra nucleotides (16) were found in the 5' end of the sequenced samples when aligned and compared to the same region from the sequences from Genbank.

Sequences from new viruses named, *Carrot chordovirus 1* and *Carrot chordovirus*

2, CtChV1 and CtChV2 (KF533711 and KF533710), from the genus *Chordovirus* were also found in samples from Yorkshire and Norfolk. Sequences from the viruses associated with the Carrot mottle dwarf disease (CMD), CtRLV, CRLaVRNA and CMoV were also identified. Sequences obtained from sample 14 had 96 % aminoacid identity to *Carrot necrotic dieback virus* (ACJ04421), a virus belonging to the *Sequivirus* genus and closely associated with *Parsnip yellow fleck virus* (PYFV). Finally, a sequence of 1524 bp corresponding to a virus from the genus *Caulimovirus* was identified although no more information could be elucidated.

Table 3.3: Sequencing results from the UK are represented as percentages of RPKM (Reads per Kilobase per million mapped reads). A percentage of 100 % indicates the presence of the whole viral sequence in that sample. RPKM values for other samples are then represented as percentages of the presence of the viral sequence compared to the whole sequence (100 %).

		Stamford Bridge					Sutton on the Forest				Elveden					Hingham "high risk"					Hingham "low risk"					
Virus	**Size (bp)**	**S1**	**S2**	**S3**	**S4**	**S5**	**S6**	**S7**	**S8**	**S9**	**S10**	**S11**	**S12**	**S13**	**S14**	**S15**	**S16**	**S17**	**S18**	**S19**	**S20**	**S21**	**S22**	**S23**	**S24**	**Pos**
CMoV	4261	57%	2%	18%	49%	24%	5%	13%	8%	4%	100%	28%	24%	79%	41%	60%	35%	45%	21%	31%	18%	26%	12%	26%	10%	4%
CMoV	3571	8%	0%	0%	0%	2%	0%	0%	1%	0%	12%	57%	35%	51%	43%	100%	93%	70%	17%	93%	51%	15%	19%	18%	100%	0%
CtRLaVRNA	2837	0%	0%	0%	0%	0%	0%	0%	0%	1%	12%	41%	43%	89%	47%	100%	97%	94%	11%	70%	53%	9%	26%	1%	64%	0%
CtRLaVRNA	2825	0%	0%	0%	0%	0%	0%	0%	0%	0%	2%	1%	3%	18%	100%	6%	13%	12%	1%	12%	2%	1%	0%	0%	1%	0%
CtRLV	4109	0%	1%	0%	0%	1%	1%	1%	1%	0%	5%	18%	14%	28%	29%	44%	42%	96%	8%	20%	35%	11%	5%	42%	100%	0%
CtRLV	4064	0%	0%	0%	1%	1%	0%	1%	0%	0%	7%	21%	15%	35%	23%	59%	44%	48%	10%	25%	22%	12%	6%	37%	100%	0%
CaTV1	6883	0%	0%	0%	0%	0%	0%	0%	0%	0%	14%	5%	23%	10%	8%	100%	33%	6%	18%	17%	0%	0%	0%	0%	1%	0%
CaTV1	6835	0%	2%	0%	0%	0%	0%	0%	2%	3%	1%	24%	0%	3%	0%	3%	3%	6%	9%	0%	1%	100%	0%	1%	1%	0%
CaTV1	4757	0%	0%	0%	0%	0%	1%	0%	0%	1%	15%	15%	34%	60%	42%	100%	52%	88%	23%	62%	0%	3%	0%	0%	0%	0%
CaTV1	4671	0%	1%	0%	0%	0%	0%	0%	0%	3%	11%	9%	17%	18%	32%	21%	28%	42%	16%	35%	0%	100%	0%	0%	1%	0%
CYLV	16360	0%	2%	0%	0%	0%	0%	1%	0%	1%	0%	39%	7%	8%	34%	1%	31%	100%	1%	21%	14%	0%	0%	0%	14%	0%
CYLV	16358	2%	1%	0%	0%	0%	0%	1%	0%	0%	0%	20%	1%	6%	23%	1%	16%	43%	0%	11%	0%	0%	0%	0%	100%	0%
CtCV1	6409	0%	0%	0%	1%	1%	1%	0%	0%	0%	21%	4%	1%	1%	63%	100%	4%	1%	1%	3%	1%	0%	1%	60%	1%	0%
CtCV1	5434	0%	7%	0%	0%	4%	0%	0%	0%	0%	12%	7%	0%	0%	100%	31%	0%	3%	1%	0%	1%	2%	1%	88%	3%	0%
CtChV1	1957	0%	0%	0%	0%	0%	0%	1%	0%	0%	6%	18%	0%	1%	2%	4%	100%	37%	1%	2%	1%	1%	2%	1%	2%	0%
CtChV2	2992	0%	0%	0%	0%	0%	0%	58%	3%	2%	69%	7%	0%	0%	0%	5%	0%	36%	100%	0%	4%	1%	1%	2%	8%	0%
CtChV2	7428	0%	0%	0%	0%	0%	0%	84%	0%	5%	65%	0%	0%	2%	4%	4%	4%	73%	29%	6%	100%	1%	1%	2%	7%	0%
CNDV	3239	0%	0%	0%	0%	0%	1%	2%	2%	0%	0%	1%	0%	1%	100%	0%	2%	1%	0%	2%	0%	0%	0%	75%	1%	0%
CNDV	1133	0%	1%	0%	0%	1%	1%	7%	12%	1%	0%	0%	0%	0%	73%	1%	2%	0%	0%	78%	1%	0%	0%	100%	1%	0%
PopMV	5405	0%	0%	0%	0%	0%	0%	0%	0%	1%	14%	0%	3%	1%	51%	100%	85%	5%	2%	2%	2%	20%	0%	1%	2%	0%
Caulimovirus	1524	0%	69%	0%	0%	3%	5%	62%	11%	53%	35%	17%	11%	20%	35%	45%	66%	91%	12%	49%	53%	12%	9%	18%	100%	0%

CMoV**: *Carrot mottle virus*; **CRLaVRNA**: Carrot red lead associated RNA**; CtRLV**: *Carrot red leaf virus*; **CaTV1**: *Carrot torrado virus 1*; **CYLV**: *Carrot yellow leaf virus; CtCV1**: Carrot closterovirus 1; **CtChV1**: *Carrot chordovirus 1;* CtChV2: *Carrot chordovirus 2;* **CNDV**: *Carrot necrotic dieback virus*; **CTLaV**: Cherry twisted leaf associated virus; **PopMV**: *Poplar mosaic virus*;

3.4 Discussion

In the described study, field samples of carrots collected in the UK and France have been subjected to NGS to study viral communities. The technique has been proven before as a valuable approach for the study of the virome of a particular host and discovery of novel viruses or mutant strains and it has been also successfully used for the identification of viruses which failed routinely detection procedures (Adams *et al*., 2009; Kreuze *et al*., 2009; Coetzee *et al*., 2010; Candresse *et al*., 2014).

Results indicated the presence of a novel virus from the *Carlavirus* genus in two of the samples from the UK. Although the complete genome sequence was not obtained, the sequence was compared with a similar sequence previously isolated from Cow Parsley. Following the International Committee of Taxonomy of Viruses (ICTV) demarcation criteria for species differentiation established in the 9th report, species from the genus *Carlavirus* have less than about 72 % nt identity between their polymerase or coat protein region (King *et al*., 2012). The coat protein region of the Cow Parsley isolate was seen to have 82.4 % of nucleotide identity with Carrot Carlavirus, which is above the criteria established for the *Carlavirus* genus. This species was found in carrots in Germany during a survey for viruses infecting *Apiaceae* (Menzel *et al*., 2008a). A sequence of 2235 nucleotides was then described corresponding to the 3' end and including the triple gene block proteins typical from Carlaviruses (King *et al*., 2012), but no further comparisons with any other virus could be established. Further studies are needed in order to confirm the similarity between the carrot and Cow parsley forms and to establish similarities/differences between the obtained sequence and the virus described in Germany. The presence of this new virus in the samples should be also confirmed using a second detection method such as RT-PCR by the design of specific oligonucleotides for the assembled sequence or using developed *Carlavirus* Group PCR Primer Mix (PCR 93000/0025, Agdia) (Maroon and Zavriev, 2002). The development of a specific detection

method would allow the detection and assessment of prevalence in larger numbers of samples from the field.

Sequences corresponding to *Carrot necrotic dieback virus* (CNDV) were also found in several samples from Norfolk. This virus had been initially considered as the *Anthriscus* strain of PYFV but differences found in the coat protein region and the presence of a polyA tail led to the proposal that this virus as a new species within the genus *Sequivirus* in 2009 (Van Dijk *et al*., 1985; Menzel *et al*., 2008b). It is likely that the virus has been present in the crop for a while but has been treated as infection with PYFV.

NGS has been successfully proven to be a powerful tool for the study of viral communities in carrot crops. However, one of the main limitations of this technique is still associated with the cost per run, although the advent of more affordable sequencing platforms and a competitive market have decreased the cost considerably in recent years. Also, the analysis and comparison of the assembled sequences depends on the published information available in databases such as Genbank which might also limit the outcome of the analysis if similar sequences have not been discovered or published. The challenge now is to develop easier, more efficient and quicker methods which allow NGS to be adopted in diagnostic laboratories as well as expand its use in academic research (Voelkerding *et al*., 2009).

Chapter 4

Detection and transmission of *Carrot torrado virus 1*, a novel putative member of the *Torradovirus* genus

Rozado-Aguirre, Z., Adams, I., Collins, L., Fox, A., Dickinson, M., Boonham, N. (2016). Detection and transmission of Carrot torrado virus, a novel putative member of the Torradovirus genus. Journal of Virological Methods, Vol 235, 119-124.

In this publication, we described the development of a RT-qPCR method for the detection of *Carrot torrado virus1* (CaTV1). The method was validated according to the requirements established in the European Plan Protection Organization (OEPP/EPPO Bulletin PM7/98). Also this publication, showed the first report of aphid transmission of a member of the *Torradovirus* genus.

The PhD candidate designed the primers and performed all the assays required for the development and validation of the detection method. She also helped in the transmission experiments leaded by Larisa Collins and wrote the manuscript.

Abstract

A new *Torradovirus* tentatively named *Carrot torrado virus1* (CaTV1) was an incidental finding following a next generation sequencing study investigating internal vascular necrosis in carrot. The closest related viruses are *Lettuce necrotic leaf curl virus* (LNLCV) found in the Netherlands in 2011 and Motherwort yellow mottle virus (MYMoV) found in Korea in 2014. Primers for reverse transcriptase-PCR (RT-PCR) and RT-qPCR were designed with the aim of testing for the presence of virus in plant samples collected from the field. Both methods successfully amplified the target from infected samples but not from healthy control samples. The specificity of the CaTV1 assay was also checked against other known carrot viruses and no cross-reaction was seen. A comparative study between methods showed RT-qPCR was the most reliable method, giving positive results in samples where RT-PCR fails. Evaluation of the Ct values following RT-qPCR and a direct comparison demonstrated this was due to improved sensitivity. The previous published *Torradovirus* genus specific RT-PCR primers were tested and shown to detect CaTV1. Also, virus transmission experiments carried out suggest that unlike other species of the same genus, *Carrot torrado virus1* could be aphid-transmitted.

4.1 Introduction

The genus *Torradovirus*, within the family *Secoviridae* was first described to place two new viruses, *Tomato torrado virus* (ToTV) and *Tomato marchitez virus* (ToMarV) (Sanfaçon *et al*., 2009; Verbeek *et al*., 2007; Verbeek *et al*., 2008). More recently, new species have also been proposed as members of the genus: Tomato chocolàte virus (ToChV), Tomato chocolàte spot virus (ToChsV), *Lettuce necrotic leaf curl virus* (LNLCV), Motherwort yellow mottle virus (MYMoV) and Cassava torrado-like virus (CsTLV) (Verbeek *et al*., 2010; Batuman *et al*., 2010; Verbeek *et al*., 2013a; Seo *et al*., 2014; Carvajal-Yepes *et al.,* 2014). ToTV is considered the type species of the genus, it was first found in Spain in 2004 and since then, isolates have been described all around the world (Van der Vlugt *et al*, 2015). Analysis of its genome structure indicates there are two (+) ssRNA of ~7.7 kb (RNA1) and ~5.2 kb (RNA2) (Verbeek *et al*., 2007; Budziszewska *et al*., 2008). RNA1 contains one open reading frame (ORF) and has coding regions for the protease (Pro), helicase (Hel) and RNA-dependent RNA polymerase (RdRp). RNA2 has two ORFs: The function of ORF1 is still unclear whilst ORF2 encodes three coat proteins (Vp35, Vp26 and Vp23) and the movement protein (MP). Specific primers were designed for the detection of the ToTV (Pospieszny *et al*., 2007) and two generic primer sets, Torrado-1F/Torrado-1R and Torrado-2F/Torrado-2R, were also designed for the detection and identification of all the other viruses in the genus (Verbeek *et al*., 2012). Additional sets of primers have been recently developed to detect ToTV isolates from different sources (Herrera *et al*., 2015). Previous studies showed that torradoviruses are transmitted by the whiteflies *Trialeurodes vaporariorum, Trialeurodes abutilonea* and *Bemisia tabaci,* suggesting also that they are transmitted in a semi-persistent and stylet-borne manner (Amari *et al*., 2008, Barajas-Ortiz *et al*., 2013; Verbeek *et al*., 2013b). However, experiments with LNLCV and whiteflies using the same acquisition and inoculation periods did not lead to virus transmission suggesting another vector species could be responsible (Verbeek *et al*., 2013b).

Internal necrosis has been seen in carrots (*Daucus carota*) in the UK for at least ten years and has been associated with virus infection (Fox, 2011). Recent analysis using next generation sequencing (NGS), found that *Carrot yellow leaf virus* (CYLV) was strongly associated with the development of internal necrosis symptoms in carrots (Adams *et al*., 2014). Several novel viruses were also identified in that study including a new torrado virus tentatively named as *Carrot torrado virus1* (CaTV1) (KF533719 and KF533720). Analysis of sequence data showed the closest related virus in the genus *Torradovirus* was LNLCV (Adams *et al*., 2014). The acronym CaTV1 for *Carrot torrado virus1* was later proposed in order to avoid confusion with *Carrot tristeza virus* (CTV) (van der Vlugt *et al*., 2015).

This study describes the development of a rapid and reliable molecular detection method for the identification of the first *Torradovirus* found in carrots, *Carrot torrado virus1*, using reverse-transcriptase (RT)-PCR and RT-qPCR and describes the first report of aphid transmission of one member of the *Torradovirus* genus.

4.2 Methods

4.2.1 Source of samples

Carrot leaves exhibiting a range of foliar symptoms (interveinal chlorosis, generalised chlorosis, tip reddening and tip necrosis) and asymptomatic leaves were taken from Elveden Estate Field, Thetford, Norfolk, UK (Latitude 52.3656, Longitude -0.56407). Weed samples from the Apiaceous family, assumed to be more likely infected with carrot viruses, were also taken from the margins of Bratleys Field, Stamford Bridge, Yorkshire, UK (Lat. 53.9992, Long. -0.8855) and Sutton Park field, Sutton on the Forest, Yorkshire, UK (Lat. 54.0577, Long. -1.0518). ToTV infected plants were kindly supplied by the Plant Protection Service in the Netherlands for comparative testing.

4.2.2 Nucleic Acid extraction

Carrot leaves and weed samples were extracted using the Kingfisher® mL system (Thermo labsystems) following the method described in (Mumford, 2002 and Mumford *et al*., 2004). Leaf material was ground in lysis buffer and centrifuged at 13000 rpm for 1 min. Samples were then loaded into the instrument and the extraction protocol was followed as described. RNA was eluted in 200 µl of molecular grade water and stored at -20 °C.

4.2.3 Conventional RT-PCR assay setup

Two pairs of CaTV1 primers were designed using Primer Express 2 (Applied Biosystems) for a RT-PCR assay according to the sequencing data obtained by Adams *et al*. (2014) (Table 1). The primers designed to RNA1 amplify a fragment of 262 bp and RNA2 primers a fragment of 299 bp. The extracted sample (1 µl) was added to a 24 µl reaction mix, containing Verso™ 1-Step RT-PCR ReddyMix™ Kit (Thermo Scientific)

and 400 mM of each primer. Assays were carried out in a Bio-Rad C1000^{TM} thermal cycler (Bio-Rad laboratories) and PCR conditions consisted of 45 min at 48 °C for cDNA synthesis, 2 min at 94 °C, then 40 cycles of, 30 sec at 94 °C, 1 min at 56°C and 1 min at 68 °C and a final extension step for 6 min at 68 °C. These conditions followed the method described in Verbeek *et al*. (2012), but primer annealing temperature of 56 °C was chosen for CaTV when a gradient primer annealing temperature test was performed on a CFX96 Touch Thermal cycler (Bio-Rad laboratories) for optimization. PCR products were separated using a 1.8 % agarose gel (130 V) stained with ethidium bromide, visualized in a UV transilluminator. Products were purified using the QIAquick® PCR Purification kit (Qiagen) before being sent for sequencing. Generic *Torradovirus* genus assays were performed following the method described in Verbeek *et al*. (2012).

4.2.4 One step reverse-transcriptase (RT)-qPCR assay

Development of a RT-qPCR assay for CaTV1 detection

Primers and probes for the real-time assay were designed using Primer Express 2 (table 4.1). The 5' and 3' ends of the probes were labelled with the reporter dye FAM (6-carboxyfluorescein) and quencher dye TAMRA (tetra-methylcarboxyrhodamin). A concentration of 300 nM of each primer and 100 nM of probe were used in each 25 µl reaction and same conditions were used for both RNAs. Reactions were carried out in 96 well plates using the ABI 7900 (Applied Biosystems). Positive controls consisting of a CaTV1 sample obtained by NGS and negative controls consisting of healthy leaf material and water were used to validate the results. RT-qPCR cycling conditions were: 10 min at 55 °C for the reverse transcription followed by 8 min at 95 °C and then 40 cycles of 10 sec at 95 °C and 1 min at 60 °C. Results were analysed using the SDS 2.4 Software (Applied Biosystems). A threshold cycle (Ct) value below 40 was considered as a positive result and

was fixed by default parameters of the Software.

Table 4.1: RT-PCR and RT-qPCR *Carrot torrado virus1* (CaTV1) RNA1 and RNA2 primers used during the study.

	Primer	Sequence (5'-3')	Location (nt)
RT-PCR	**CaTVPCR 1F**	TCAATCAGTATTAAGCGAGGAATGG	2742 - 2762
	CaTVPCR 1R	CCTCAATGGGCTTGTAATGA	2985 – 3004
	CaTVPCR 2F	TGTGCAACCACGAGGAATACA	3942 – 3962
	CaTVPCR 2R	GATGCCTCATAGCAAACTGTCAT	4219 - 4241
RT-qPCR	**CaTV-1F**	CCGTTGTTATTCGTCTTCCTCAA	2819 – 2841
	CaTV-1R	TGGATGATTGTAAATACTGCACCAT	2918 - 2942
	CaTV-1P	FAM-TTCAGAGGTGTTTACGTGAGATCGGGATG-TAMRA	
	CaTV-2F	TTACAAAGACTACTGGTGATCGTGACTT	2654 – 2681
	CaTV-2R	ATTCGTACAAACCCACCTCAAAG	2730 - 2752
	CaTV-2P	FAM-GAGTTGGAAATGATGCAACCCATGATAGC-TAMRA	

Validation experiments

The efficiency of the new assays for both RNAs was measured using serial 10-fold dilutions (from 1 to 10^{-8}) of total RNA from plant infected extracts quantified using the Nanodrop ND-1000 Spectrophotometer (Labtech). Samples were chosen from a pool of positive samples previously tested using RT-qPCR and all the dilutions were tested in duplicate in the same run. Standard curves were generated using the Ct values obtained and the logarithm of the dilution and regression coefficient represented. Specificity of the real-time test was assessed using a panel of carrot and other viruses. Analytical sensitivity was also compared to a RT-PCR assay comparing RT-qPCR Ct values with band intensity of the PCR product after gel electrophoresis.

4.2.5 Transmission experiments

Infected leaves were ground in potassium phosphate buffer, pH 7.7., mixed with cellite and inoculated onto leaves of ten plants of *Nicotiana benthamiana, Anthriscus cerefolium* (chervil) and *Daucus carota* (carrot). Plants were kept in the green house with a 12 hours photoperiod and an average temperature between 18-20 °C. Five mock inoculated plants of each species were also used as controls and kept in the same conditions. All plants were assessed weekly for symptom development and tested for virus presence using RT-qPCR from random leaves.

Cavariella aegopodii and *Myzus persicae* from a CaTV1 infected plant from the field were collected and cultured on chervil and carrot plants in a glasshouse (20 °C, 60 % RH, L16h: D8h with supplemental lighting). Aphids were then used to inoculate 3x50 trays of healthy cotyledon stage *N. benthamiana*, chervil and carrots. Healthy plants of each species were also used as negative controls. Each aphid was transferred onto an individual indicator plant and covered with a plastic tube (30 mm dia. x 110 mm) to prevent it from escaping. The tubes were removed after 24h and the aphids killed by spraying the plants with Bug Clear Ultra (0.05 gl-1 acetamiprid, The Scotts Company (UK) Ltd), following the manufacturer's instructions. The plants were grown on in a plant growth room (20 °C, 60 % RH, L16h: D8h) for three weeks when they were tested for virus presence using RT-qPCR.

4.3 Results

4.3.1 Diagnostic performance of RT-PCR

Comparison of primer sequences to sequences in Genbank using BLAST did not indicate significant homology with any other species except the targets. Assays for RNA1 and RNA2 were evaluated using extracts of carrot leaf samples from the field. Amplification products were analysed using agarose gel electrophoresis and results showed a single amplicon of the desired size for both primer sets, 262 bp for RNA1 and 299 bp for RNA2 (figure 4.1a). RT-PCR products were purified and sequenced confirming CaTV1 virus presence. No PCR products were amplified from the healthy or water controls and primer pairs did not produce non-specific amplicons.

Specific *Torradovirus* genus primers developed by Verbeek et al., (2012) were evaluated for the detection of CaTV1 and compared with the species specific assays. As expected the Generic set of primers detected an amplicon of 514 bp from CaTV1 and 515 from ToTV (figure 4.1b). The CaTV1 assay developed did not cross react with ToTV, similarly, the ToTV assay did not amplify CaTV1. Comparative analysis of the sequences using MEGA 6 did not indicate cross-reaction with LNLCV or MYMoV, non-tomato Torradoviruses and the closest related viruses to CaTV1

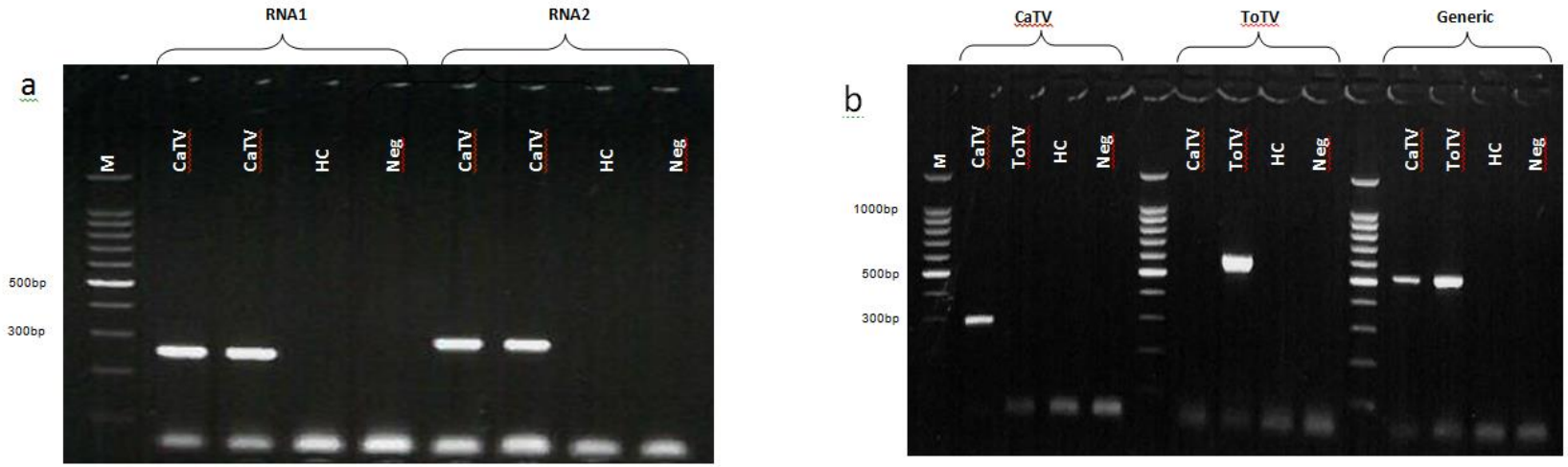


Figure 4.1: (a) RT-PCR products for RNA1 (266 bp) and RNA2 (299 bp) using *Carrot torrado virus1* (CaTV1) specific primers. (b) CaTV1 and *Tomato torrado virus* (ToTV) gel bands obtained when CaTV1, ToTV and *Torradovirus* genus sets of primers were used.

4.3.2 Detection of CaTV1 by RT-qPCR

Each primer sequence was compared to published sequence information available and no cross-reaction was found (NCBI, BLAST). The RT-qPCR assay was evaluated on an ABI 7900 HT system (Applied Biosystems) using samples collected from the field. Amplification curves gave Ct values between 15.98 and 33.19 for RNA1 and 17.02 and 32.69 for RNA2, indicating virus presence in the samples. A test designed to detect the cytochrome oxidase gene sequence (COX) of the plant was also used as an internal control. Detection of one of the two RNAs was considered as a positive result. Negative controls consisting of RNA extracts from healthy plants extracts and water were used and no amplification was detected.

To test the specificity of the assays, a range of UK field isolates affecting carrots, related and unrelated viruses were used: ToTV (*Tomato torrado virus*), CYLV (*Carrot yellow leaf virus*), CtRLV (*Carrot red leaf virus*), CtRLaVRNA (Carrot red leaf associated RNA), CMoV (*Carrot Mottle virus*), CtCV1 (Carrot closterovirus 1), PYFV (*Parsnip yellow fleck virus*), CMV (*Cucumber mosaic virus*), SLRV (*Strawberry latent ringspot virus*), TBRV (*Tomato black ring virus*) and PVY (*Potato virus Y*). Each assay was run in triplicate for every virus and no amplification was found between the species tested or the healthy or negative controls for RNA1 and RNA2 (Table 4.2). For RNA1, a Ct value of 38.12 and 39.9 was obtained for CYLV and CMoV respectively in one of the replicates. Those samples were tested again and no amplification was seen.

4.3.3 RT-qPCR assay validation and sensitivity comparison with RT-PCR

In order to assess the efficiency of the assays, standard curves for RNA1 and RNA2 were generated with 10-fold serial dilutions of total RNA (plant + virus) from a pool of positive samples to determine the maximum dilution detected. For each dilution, two replicates with 1 µl of total RNA were prepared in a 25 µl well. Results showed standard curves with 0.9932 and 0.9956 correlation coefficients (r^2) for RNA1 and RNA2 respectively (figure 4.2a). Both assays were assessed following the guideline described in OEPP/EPPO Bulletin PM7/98 (2) (2014) (Table 4.3). Repeatability tests were performed for both RNAs with the lowest level of dilution detected reliably in the sensitivity assays and six replicates of each sample were tested. Ct values between 33.91 and 35.17 were obtained with a standard deviation (SD) of ± 0.46 for RNA1 and between 32.15 and 32.63 with a SD of ± 0.19 for RNA2 were obtained. No differences were seen either when tested by two different operators in two different 7900 HT systems.

Diagnostic sensitivity of the RT-qPCR assay was also compared to RT-PCR. Results showed that the developed RT-qPCR was more sensitive than the conventional method detecting dilutions ranging between 19 ng and 1.9 pg and detecting levels of dilution up to 10^{-5} for RNA1 and 10^{-4} for RNA2 (Figure 4.2b), 1000 and 10^4 times more sensitive than the conventional method which detected levels of dilution of 10^{-1} in both RNAs (Figure 4.2c). Further comparisons were made between both methods using a pool of 45 samples collected from the field. For RNA1, RT-qPCR detected 23 positive samples (51.1 %) while RT-PCR detected virus presence in a total of 20 samples (44.4 %) (data not shown). When tested for RNA2, RT-PCR gave positive results in 27 of the samples (60 %) in comparison with the 31 positives obtained by RT-qPCR (68.8 %) (Figure 4.2d).

Table 4.2: *Carrot torrado virus1* (CaTV1) RT-qPCR assay specificity was tested against *Tomato torrado virus* (ToTV), carrot viruses and non-target viruses*. Results are represented as the number of RT-qPCR cycle (Ct) at which amplification was observed in a 40 cycles assay.

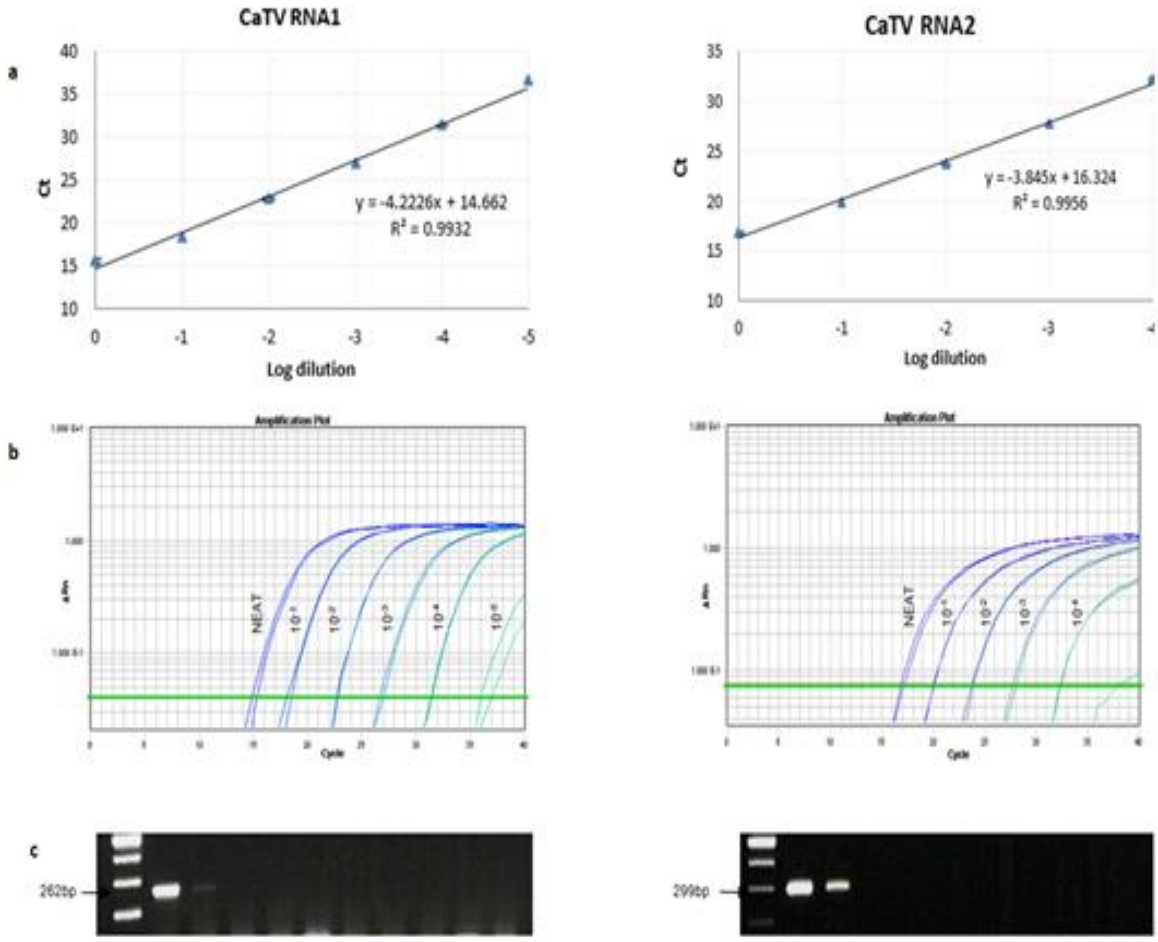
	CaTV1	ToTV	CYLV	CTRLV	CRLaV	CMoV	CtCV1	PYFV	CMV	SLRV	TBRV	PVY	HC
RNA1 Ct	15.98 ± 0.0 (3/3)	-	38.12 (1/3)**	-	-	39.9 (1/3)**	-	-	-	-	-	-	-
RNA2 Ct	17.47 ± 0.14 (3/3)	-	-	-	-	-	-	-	-	-	-	-	-

*ToTV (Tomato torrado virus), CYLV (*Carrot yellow leaf virus*), CTRLV (*Carrot red leaf virus*), CRLaV (*Carrot red leaf associated virus*), CMoV (*Carrot mottle virus*), CtCV1 (Carrot closterovirus 1), PYFV (*Parsnip yellow fleck virus*), CMV (*Cucumber mosaic virus*), SLRV (*Strawberry latent ringspot virus*), TBRV (*Tomato black ring virus*), PVY (*Potato virus Y*).

** Samples tested negative when assay was repeated, indicating there was no cross-reaction with any other viruses.

Table 4.3: Validation results obtained for *Carrot torrado virus 1* (CaTV1) RNA1 and RNA2 assays following the criteria described in OEPP/EPPO Bulletin PM 7/98 (2).

	CaTV RNA1	**CaTV RNA2**
Sensitivity		
Ct value obtained for the smallest amount of target detected reliably	36.48 ± 0.66	33.6 ± 1.46
Specificity		
Cross reacts with	-	-
Repeatability		
Calculated % of agreement for a low concentrated sample (10^{-5} dilution)	100 %	100 %
Ct	34.33 ± 0.46 (33.91-35.17)	32.43 ± 0.19 (32.15-32.63)
Reproducibility		
Calculated % of agreement for a low concentrated sample (10^{-4} dilution)	100 %	100 %
Operator 1:		
7900 HT system 1 Ct	34.93 ± 0.49	33.34 ± 0.23
7900 HT system 2 Ct	34.77 ± 0.55	33.38 ± 0.83
Operator 2:		
7900 HT system 1 Ct	34.97 ± 0.44	31.99 ± 0.11
7900 HT system 2 C	34.66 ± 0.40	31.91 ± 0.27



d

		RT-PCR		
		+	-	**Total**
RT-qPCR	+	27	4	31 (68.9 %)
	-	0	14	14 (31.1%)
	Total	27 (60 %)	18 (40 %)	45 (100 %)

Figure 4.2: RT-qPCR standard curves and linear correlation coefficients (r^2) for *Carrot torrado virus1* (CaTV1) RNA1 and RNA2 using serial 10-fold dilutions of total RNA with primers described in table 1 (2a). Amplification plots obtained using RT-qPCR (2b). Gel bands obtained with CaTV1 serial diluted samples using RT-PCR (2c). Comparative test between RT-qPCR and RT-PCR using a pool of 45 samples collected from the field (2d).

4.3.4 CaTV1 transmission

Mechanical transmission experiments with *N. benthamiana*, chervil and carrots resulted in one symptomless *N. benthamiana* CaTV1 infected plant when tested using RT-qPCR.

Weed samples surrounding carrot fields consisting of 30 cow parsley (*Anthriscus sylvestris)*, and 19 hogweed (*Heracleum sphondylium),* were collected and tested to look for possible sources of CaTV1 infection in carrots but all tested negative for the virus (data not shown).

Aphid transmission experiments with *M. persicae* and *C. aegopodii* resulted in some symptomatic chervil and *N benthamiana* plants when tested by RT-qPCR. Transmission rates of 35.3 % and 12.7 % were found in chervil and tobacco plants respectively with *M. persicae*. Lower transmission rates were achieved when using *C.aegopodii* in both plant species (Table 4.4). Carrot to carrot transmission of 10 % and 2.7 % was also seen with *M. persicae* and *C. aegopodii* respectively. Virus was successfully transmitted from infected Chervil to healthy carrots using *M. persicae* and 2 % of the plants tested positive for the virus. All the negative controls consisting of healthy carrot, *N. benthamiana* and chervil plants were also used for each experiment and tested negative for CaTV1.

Table 4.4: Results of *Carrot torrado virus 1* (CaTV1) transmission experiments using *Cavariella aegopodii* and *Myzus persicae* aphid species from chervil and carrot to *Nicotiana benthamiana*, chervil and carrot. Samples were tested using RT-qPCR.

Source	Species	*M. persicae*		*C. aegopodii*	
		Positives	**% transmission**	**Positives**	**% transmission**
Carrot (*D. carota*)	*N. benthamiana*	10/50 2/50 7/50	12.7	0/0 0/0 0/0	0
	Chervil	18/50 13/50 23/50	35.3	3/50 0/50 4/50	4.7
	Carrot	5/50 6/50 4/50	10	1/50 2/50 1/50	2.7
Chervil (*A.cerefolium*) back transfer	Carrot	1/50 2/50 0/50	2	-	-



Figure 4.3: Chervil (*Anthriscus cerefolium*) plants positive for *Carrot torrado virus 1* (CaTV1). Plants showed stunting and interveinal chlorosis.

4.4 Discussion

CaTV1 was firstly detected in the UK in 2013 in a study investigating the agent responsible of the development of necrotic symptoms in carrots (Adams *et al*., 2014). In order to detect CaTV1 infected plants from the field and study its transmission, RT-PCR and RT-qPCR assays were developed and optimized. Both methods are routinely used in diagnostic laboratories for the detection of pathogens. In this study both methods successfully amplified CaTV1 RNA1 and RNA2 in infected samples collected from the field. Neither assay cross-reacted with ToTV, the first *Torradovirus* found and type member of the genus, indicating the specificity of the assays. Several studies had been performed previously using RT-PCR and two generic primer sets were developed for the detection of

all the viruses of the *Torradovirus* genus (Verbeek *et al*., 2012). These sets of primers were evaluated and the detection of CaTV1 RNA1 and CaTV1 RNA2 using the same cycling conditions was confirmed. Assays were subjected to the EPPO validation international standard method and all the requirements were met. Validation experiments using RT-qPCR were carried out and it was seen to detect virus levels up to 1.9 pg. In specificity assays, samples with Ct values between 36 and 40 were re-tested to confirm if there was cross-reaction or low level contamination due to late amplification of some random samples. Re-testing confirmed there was no cross-reaction with any of the species tested. Similar results were obtained when the assays were performed by different people, different days in different 7900 HT systems indicating the reproducibility and repeatability for both RNAs and the robustness of the developed assays. RT-qPCR method gave positive results in field samples where RT-PCR failed and further comparison between both methods indicated this was due to improved sensitivity.

Some samples were found to be only positive for RNA1 but negative for RNA2 and vice-versa when tested using RT-PCR or RT-qPCR, indicating possible replication differences between RNAs when infection takes place in the host. The performance of the tests for both RNAs is recommended to avoid any false negative result.

CaTV1 was successfully inoculated and transmitted to healthy *N. benthamiana* and Chervil plants but symptom description was not possible due to infection with *Carrot red leaf virus* too. Previously studies suggested that tomato infecting *Torradoviruses* were transmitted by different whiteflies species (Amari *et al*., 2008; Verbeek *et al*., 2013b). However, aphid transmission experiments developed in the glasshouse with CaTV1, indicated that *M. persicae* could be the most likely vector of the virus to carrots. Equally, LNLCV did not result in transmission when experiments with whiteflies were carried out suggesting there could be another different species involved (Verbeek *et al*., 2013). These results conclude the possibility that non-tomato infecting Torradoviruses are transmitted by different species than tomato infecting Torradoviruses. Back transmission experiments also

showed the virus can be transmitted between members of the *Apiaceae* family.

In 2008, ToTV was found in weed species from *Amaranthaceae*, *Caryophyllaceae*, *Chenopodiaceae*, *Cruciferae*, *Malvaceae*, *Polygonaceae*, and *Solanaceae* families (Alfaro-Fernandez *et al*., 2008) indicating they could act as reservoir hosts for this virus before their transmission to tomatoes by whiteflies. However, limited surveys carried out in 2014 and 2015 with weeds from several species, 59 Hogweed (*Heracleum sphondylium*), 27 Cow Parsley (Anthriscus sylvestris), one Hemlock (*Conium maculatum*) and three Rough chervil (*Chaerophyllum temulum)* did not identify any alternative host for CaTV1 suggesting that infection could come from another different source (Adrian Fox, personal communication). Due to the limited number of weed samples tested from Yorkshire, further studies need to be carried out in order to establish possible sources of infection in carrots with CaTV1.

The study describes the development of a new, reliable, and sensitive RT-qPCR method for the detection of CaTV1 and the first report of aphid transmission of a member of the *Torradovirus* genus. However, additional studies are also currently being carried out evaluating CaTV1 host range, transmission and further weed testing. Symptom development and incidence of this virus in the UK will also be assessed in order to obtain a complete characterization of this new finding.

Chapter *5*

Complete sequence and genomic annotation of *Carrot torrado virus1* (CaTV1)

Rozado-Aguirre, Z., Adams, I., Fox, A., Dickinson, M., Boonham, N. (2017). Complete sequence and genomic annotation of Carrot torrado virus 1 (CaTV1). Archives of Virology. doi:10.1007/s00705-017-3410-5

In this publication, CaTV1 complete genome sequences were obtained using RACE and sequences were updated in Genbank. Genome annotation of both RNA complete sequences was carried out and comparisons against other viruses performed.

The PhD candidate performed the experiments required to obtain CaTV1 complete genome sequences. She also carried out the genome annotation work, sequence comparisons and wrote and submitted the manuscript.

Abstract

Carrot torrado virus 1 (CaTV1) is a new member of the *Torradovirus* genus within the family *Secoviridae*. CaTV1 genome sequences were obtained from a previous Next Generation Sequencing (NGS) study and were compared to other members and tentative new members of the genus. The virus is comprised of a bipartite genome and RACE was used to amplify and sequence each end of RNA1 and RNA2. As a result RNA1 and RNA2 are estimated as comprising 6944 and 4995 nucleotides respectively, with RNA1 encoding the proteins involved in virus replication, and RNA2 encoding the encapsidation and movement proteins. Sequence comparisons showed that CaTV1 clustered within the non-tomato infecting torradoviruses and is most similar to *Motherwort yellow mottle virus* (MYMoV). The nucleotide identities of the Pro-Pol and coat protein regions were below the criteria established by the ICTV for demarcating species, confirming that CaTV1 should be classified as a new species within the *Torradovirus* genus.

5.1 Introduction

Carrot torrado virus 1 (CaTV1) was an incidental finding discovered in a Next Generation Sequencing (NGS) study seeking to elucidate the causes of internal necrosis in carrots (*Daucus carota*) in the UK (Adams *et al*., 2014). CaTV1 has been recently reported in carrot leaves collected in the Southwest of France, indicating its presence also outside the UK (Rozado-Aguirre *et al.,* 2017). The virus is similar to members of the *Torradovirus* genus, first described in 2007 to place two new viruses affecting tomato crops, *Tomato torrado virus (ToTV)* and *Tomato marchitez virus* (ToMarV) (Verbeek *et al.,* 2007; Verbeek *et al.,* 2008). Later more viruses affecting different crops have been added to the genus, including, Tomato chocolate virus (ToChV), Tomato chocolate spot virus (ToChSV), Tomato necrotic dwarf virus (ToNDV), *Lettuce necrotic leaf curl virus* (LNLCV), *Motherwort yellow mottle virus* (MYMoV), Cassava torrado-like virus (CsTLV) and Squash chlorotic leaf spot virus (SCLSV) (Verbeek *et al*., 2010; Batuman *et al*., 2010; Verbeek *et al*., 2013; Seo *et al*., 2014; Carvajal-Yepes *et al*., 2014*;* Lecoq *et al.,* 2016). Torradoviruses are considered members of the *Secoviridae* family within the order *Picornavirales* (Sanfacon *et al*., 2009) and previous sequence comparisons within this genus established two different clades for tomato-infecting (TI) and non-tomato infecting (NTI) members (van der Vlugt *et al*., 2015).

Although CaTV1 was a sequencing finding using NGS, the sequence of the virus was not fully described. In this study we complete the characterization of the genome of CaTV1, including completion of the 3' and 5´ends of both RNA fragments using RACE, annotation of the genome identifying the location of translational features, and establishing the similarities between this and other members of the *Torradovirus* genus.

5.2 Completion of the CaTV1 genome

The 3′ and 5′ ends of both RNA1 and RNA2 were amplified using the SMARTer RACE cDNA amplification kit (Clontech) according to the manufacturer's protocols. Primers used for both RNAs are described in Table 5.1 and were designed using Primer express 2. The 3′ and 5′ PCR products were analysed by direct sequencing. Results indicated that the 5′ UTR and 3′ UTR regions of RNA1 were 127 and 240 nt long respectively, and for RNA2, were 611 nt (5′ UTR) and 327 (3′ UTR) in length. Completed sequences were deposited in GenBank with accession numbers KF533719.2 and KF533720.2 for RNA1 and RNA2 respectively.

Table 5.1: *Carrot torrado virus 1* (CaTV1) primers used for RACE (Rapid amplification of cDNA ends) experiments.

	Primer	Sequence (5'-3')
RNA1	**CaTV1-3RACE F**	CCTCTCTCCTTCTTGGTGGTTGGATATTACTTGGAGA
	CaTV1-3RACE R	ATATCAGCGAACAGCTGAAGCGCAGCTCG
	CaTV1-5RACE F	TCTGTTGGCTGTTCTACCCTTCTTTGTAGACTGCTA
	CaTV1-5RACE R	GGAAGCTTTGGAGAGAACCTCATGAATAGGTTCC
RNA2	**CaTV2-3RACE F**	GCACCTTTTGATTTATCTACCTTCGCCAGAGCTTG
	CaTV2-3 RACE F	TCCGCTGCAGGTGTAGGTGCTCTAACTTGG
	CaTV2-5RACE F	TATCACCCCACTCCTAAGATTGCACTGCCTGCAA
	CaTV2-5RACE R	CGCGGAATTCAGTTGATTATCTTGTGTGCTGATG

5.3 CaTV1 genome organization

CaTV1 - RNA1

As a member of the *Secoviridae* family, RNA1 is likely to code for a single ORF translated into a polyprotein which is then likely to be processed by serine-like proteases into mature proteins (Sanfacon *et al*., 2009). Following analysis of RNA1, one predicted ORF (RNA1-ORF1) was identified (6944 nts), encoding a putative polyprotein of 2192 amino acids (aa) with a molecular mass of 249 kDa. The translational start (AUG) and stop (UAA) codons were found at nucleotide positions 127-129 and 6703-6705 respectively (Figure 5.1).

The complete nucleotide sequence was compared to other sequences available in GenBank using BlastN, revealing 71 % identity to LNLCV (KC855266) and ToMarV (KT756874), 69 % to ToChSV (GQ305131) and ToTV (KM091449) and 68 % to MYMoV (KM855266) and ToNDV (KC999058), all members of the *Torradovirus* genus.

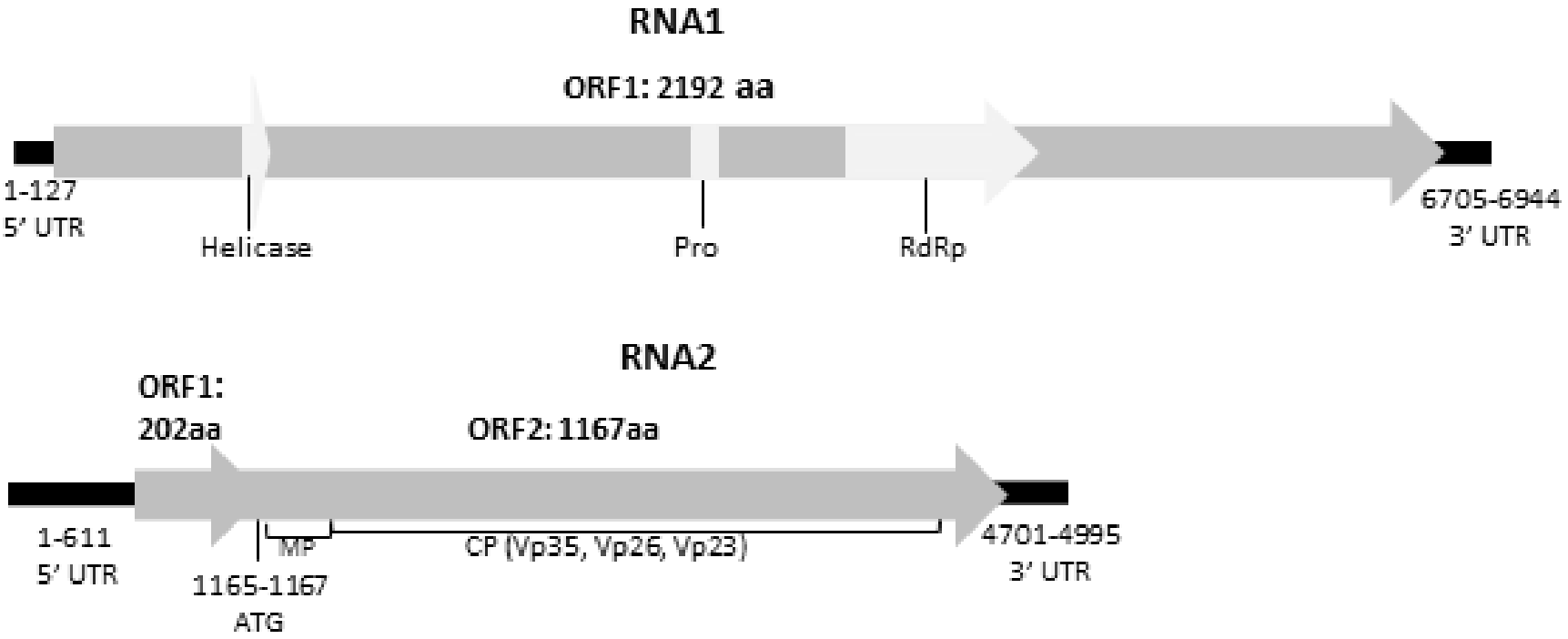


Figure 5.1: Proposed annotated diagram for *Carrot torrado virus 1* RNA1 and RNA2 sequences

Identification of protein motifs were made based on previous characterization of ToTV and ToMarV (Verbeek *et al*., 2007; Verbeek *et al*., 2008). The polyprotein contains the conserved Hel-Pro-Pol replication block typical of picorna-like viruses (nt positions 401-1516). Comparison of the aa sequence of the Pro-Pol region (1072-1516), limited by the "CG" motif of the 3C-like proteinase and the "GDD" motif of the RNA-dependent RNA polymerase (RdRp), suggested levels of similarity lower than those specified in the species demarcation criteria (< 80 %) established in the ninth ICTV report (King *et al*., 2012). This conserved domain is typically used to determine differences among different picornavirales members. Pairwise comparisons using the Hel-Pro-Pol protein sequences with other torradoviruses, showed that CaTV1 shares 58.5 %, 59.5 % and 41.2 % of the sequence with LNLCV, MYMoY and SCLSV respectively. Lower levels were found when the same region was compared to tomato-infecting torradoviruses. Typical helicase motifs (type III helicase), A (GKT), B (DD) and C (N) were found at positions 410, 456 and 507 respectively. The region, between aa 401-508, shares levels of identity up to 84 % with the corresponding region of LNLCV and 82.9 % with MYMoV, while lower levels of identity were found in the tomato-infecting torradoviruses (46.4 % ToTV and 50 % ToMarV). A histidine residue in the putative protease substrate binding pocket is located at aa position 1075 and is required for proteolytic processing in members of the *Secoviridae* family. Typical RdRp motifs (I-VII) were found between aminoacid positions 1311-1596 (Koonin, 1991). Pairwise comparison revealed that the closest amino acid identities in the RdRp domain were found with LNLCV (75.1 %) and MYMoV and ToChV (70.5 %).

To determine the relationship between CaTV1 and other viruses of the family, a neighbour-joining phylogenetic tree with 500 bootstrap replications was constructed with the RdRp region using MEGA6 and ClustalX (Figure 5.2a). The analysis revealed different clades for TI and NTI members confirming previous results described (van der Vlugt *et al*., 2015). It also indicates differences between NTI torradoviruses in the RNA1 with Squash chlorotic leaf spot virus (SCLSV), the latest proposed member of the genus, in an

independent clade.

CaTV1 - RNA2

RNA2 (4995 nt) has two predicted ORFs encoding two polyproteins. ORF1 encodes a putative polyprotein of 202 aa with a predicted molecular weight of 22 kDa (Figure 5.1). This ORF, partially overlaps the large ORF2, and is a distinguishing feature of the *Torradovirus* genus. The closest related member was MYMoV (83 % identity). No conserved motifs were found in the sequence and several differences were seen between TI and NTI torradoviruses, confirming previous results reported (van der Vlugt *et al*., 2015).

ORF2 encodes a large polyprotein of 1167 aa (130 kDa). The translational start (AUG) codon was found at nt positions 1165-1167 while the stop (UAA) codon was at nt positions 4666-4668. When the whole nucleotide sequence was compared to other members of the *Secoviridae* family, the most closely related members were LNLCV (68 % identity) and MYMoV (66 % identity).

The region encoding the movement protein (MP) domain was found between aa 45-239 with the typical LxxPxL motif in positions 211-216. Based on homology to related viruses and previous identification of ToMarV and ToChSV cleavage sites (Ferriol *et al*., 2016) the cleavage site between the MP and the first coat protein is likely to be located at position 487 (Q^{487}/A^{488}). The coat protein region shares levels of identity of 42.2 % and 47.3 % with LNLCV and MYMoV respectively, which again are the closest related members. This percentage also demarcates CaTV1 as a new species of the genus according to the criteria established in the ICTV ninth report (King *et al*., 2012). A glutamine residue (Q) at position -1 is highly conserved in all the torradoviruses and could potentially act as a protease cleavage site among the different proteins (Ferriol *et al*., 2016). By comparison with ToMarV and ToChSV cleavage sites, a Q residue has been identified at position 695 (Q^{695}/S^{696}) which could be a potential cleavage site between the Vp35/Vp26 coat proteins. Additionally, the putative Vp26 and Vp23 cleavage site region would be located at aa position 935 (Q^{935}/I^{936}). However, the actual number and size of the predicted capsid

proteins for CaTV1 have not been determined experimentally.

A phylogenetic tree was constructed with all the members of the *Torradovirus* genus using the whole region with the three coat proteins (Figure 5.2b). In agreement with the results obtained with RNA1, three different clades can be again differentiated: five viruses cluster in the tomato-infecting torradovirus clade; CaTV1 is grouped with MYMoV and LNLCV in the non-tomato infecting (NTI) torradovirus clade; and SCLSV clusters with Cassava torrado-like virus (CsTLV) sequences in a third independent clade.

CaTV1 is transmitted by aphids (Rozado-Aguirre *et al*., 2016) and a search of possible aphid transmission motifs was carried out comparing the coat protein region of TI and NTI torradoviruses. DAG (Asp-Ala-Gly) is usually a highly conserved motif in N-terminal of the coat protein of potyviruses and it has been demonstrated that the change of any of the amino acid prevents transmission by aphids (López-Moya *et al*., 1999]. However this motif could not be found within the CaTV1 sequence. TI torradoviruses have been described to be whitefly-transmitted (Verbeek *et al*., 2013; Amari *et al*., 2008), but no transmission motifs have been identified in the sequences for any of these members so far.

The presence of a small (3-5 kDa) VPg linked to the 5′ end of the RNAs has been confirmed for most members of the order; comparative genomics strongly suggests that this property is universally conserved among the genus and the *Secoviridae* family (van der Vlugt *et al*., 2015).

This study has described a complete characterization of the whole genome sequences of CaTV1 for both RNAs by comparison with other members of the genus and confirms that CaTV1 is a new species according to the criteria established by the ICTV. Phylogenetic studies using both RNAs have also confirmed and given further evidence of the differences between non-tomato infecting torradoviruses.

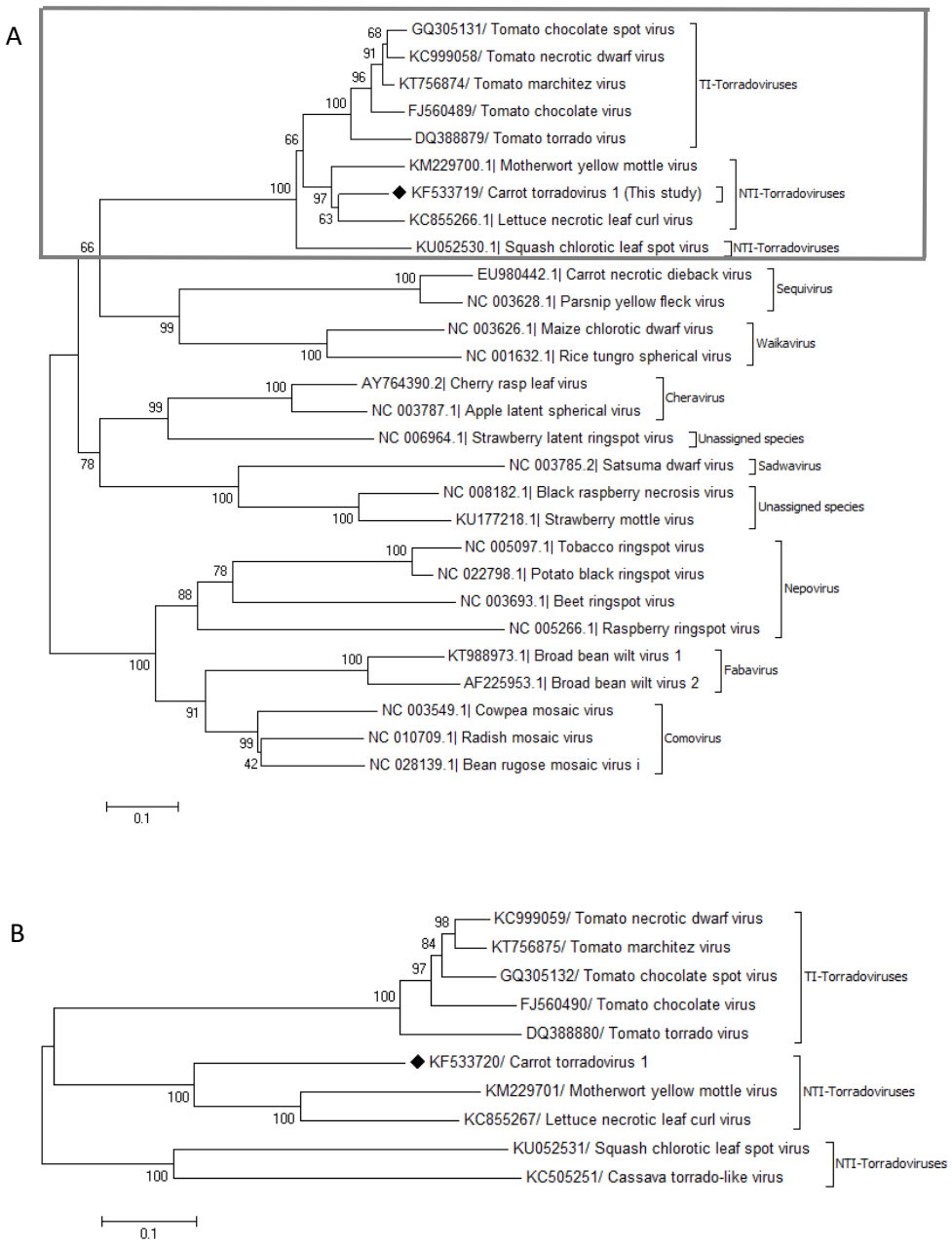


Figure 5.2: (a) A phylogenetic tree of sequences of viruses within the *Secoviridae* family was constructed using the RdRp amino acid region. (b) A second phylogenetic tree constructed using the whole coat protein region of *Carrot torrado virus* 1 RNA2 of all the members of the Torradovirus genus. Alignments were done using MEGA6 using the neighbour-joining algorithm. Sequences of all the viruses were chosen from GenBank and accession numbers are shown. The numbers at the branch points are the percentage bootstrap values following 500 bootstrap resampling and the scale indicates the number of amino acid substitutions per site.

5.3.4 Supplementary material

Pairwise comparison Hel-Pro-Pol region (RNA1)

	CaTV1	LNLCV	MYMoV	SCLCV	ToTV	ToMarV	ToChSV	ToChV	ToNDV
CaTV1									
LNLCV	0.415								
MYMoV	0.405	0.496							
SCLCV	0.588	0.656	0.619						
ToTV	0.574	0.624	0.608	0.648					
ToMarV	0.550	0.606	0.396	0.646	0.414				
ToChSV	0.560	0.617	0.445	0.656	0.420	0.149			
ToChV	0.556	0.612	0.410	0.645	0.406	0.167	0.209		
ToNDV	0.559	0.580	0.563	0.596	0.275	0.117	0.128	0.204	

RdRp (RNA dependent RNA polymerase) (RNA1)

	CaTV1	LNLCV	MYMoV	SCLCV	ToTV	ToMarV	ToChSV	ToChV	ToNDV
CaTV1									
LNLCV	0.249								
MYMoV	0.295	0.249							
SCLCV	0.395	0.378	0.430						
ToTV	0.395	0.360	0.412	0.395					
ToMarV	0.327	0.279	0.394	0.395	0.234				
ToChSV	0.327	0.264	0.377	0.412	0.234	0.047			
ToChV	0.295	0.279	0.395	0.378	0.249	0.072	0.097		
ToNDV	0.343	0.234	0.360	0.412	0.234	0.072	0.023	0.110	

Helicase (RNA1)

	CaTV1	LNLCV	MYMoV	SCLCV	ToTV	ToMarV	ToChSV	ToChV	ToNDV
CaTV1									
LNLCV	0.171								
MYMoV	0.160	0.182							
SCLCV	0.577	0.704	0.629						
ToTV	0.536	0.611	0.483	0.572					
ToMarV	0.500	0.611	0.483	0.535	0.021				
ToChSV	0.496	0.629	0.496	0.527	0.021	0.000			
ToChV	0.494	0.621	0.494	0.528	0.053	0.031	0.031		
ToNDV	0.511	0.611	0.512	0.527	0.042	0.021	0.028	0.010	

Coat protein region (RNA2)

	CaTV1	LNLCV	MYMoV	SCLCV	ToTV	ToMarV	ToChSV	ToChV	ToNDV
CaTV1									
LNLCV	0.578								
MYMoV	0.527	0.373							
SCLCV	0.953	1.048	1.035						
ToTV	0.912	0.984	1.019	1.031					
ToMarV	0.925	0.982	1.017	1.039	0.271				
ToChSV	0.904	0.965	1.012	1.071	0.271	0.148			
ToChV	0.931	1.007	1.018	1.035	0.322	0.205	0.220		
ToNDV	0.888	0.949	0.994	1.045	0.287	0.095	0.146	0.215	

CaTV1**: *Carrot torrado virus1*; **LNLCV**: *Lettuce necrotic leaf curl virus*; **MYMoV: Motherwort yellow mottle virus*; **SCLCV**: *Squash chlorotic leaf curl virus*; **ToTV**: *Tomato torrado virus*; **ToMarV**: *Tomato marchitez virus*; **ToChSV**: Tomato chocolate spot virus; **ToChV**: Tomato chocolate virus; **ToNDV**: Tomato necrotic dwarf virus.

Chapter 6

Elucidating the relation between the presence of novel black grass viruses and the development of herbicide resistance

Sabbadin, F., Glover, R., Stafford, R., Rozado-Aguirre, Z., Boonham, N., Adams, I., Mumford, R., Edwards, R. (2016). Transcriptome sequencing identifies novel persistent viruses in herbicide resistant wild-grasses. Scientific Reports, Vol 7, 41987.

In this publication the finding of novel viruses in wild grass populations is described. Virus presence was assessed in populations showing different herbicide resistance. A possible relationship between the expression of *Am*GSTF1 (marker of resistance) and the presence of the viruses is also studied.

The PhD candidate performed the RT-PCR assays for the detection of the novel viruses in different black grass populations. She also carried out Western-blot analysis from 31 plants from an herbicide resistant population.

Abstract

Novel viruses were found in a Next Generation Sequencing (NGS) study in asymptomatic black grass (*Alopecurus myosuroides*) plants. RNA from these novel viruses, Alopecurus myosuroides partitivirus 1 (AMPV1) and Alopecurus myosuroides partitivirus 2 (AMPV2) and Alopecurus myosuroides varicosavirus 1 (AMVV1), was more abundant in plants displaying non-target site based (NTSR) herbicide resistance that in herbicide susceptible plants. In this study, black grass from different populations characterized as herbicide-susceptible or -resistant were grown and assessed for the presence of the viruses. Results indicated the presence of viruses in all the populations. At the same time, the expression of the *Am*GSTF1, protein (a marker for NTSR), was evaluated in infected and uninfected plants from a NTSR population. Although no relationship could be found between the presence/absence of the viruses and the expression of *Am*GSTF1, the higher abundance of the viruses in resistant populations could suggest a role for these new viruses in the enhancement or development of resistance or, virus titre might increase in response to the changes in metabolism of the infected plants.

6.1 Introduction

Historically, viruses have usually been associated with the development of disease since they were firstly described with the discovery of *Tobacco mosaic virus* in 1898 (Beijerinck, 1898). Many virus diseases have been characterized and associated with the development of acute symptoms in plants such as necrosis, stunting and severe yield losses in crops have been described (Rodoni 2009; Bowers *et al*., 2011). However, in recent years the hypothesis of unexpected benefits due to virus infection has already been proposed by some researchers (Roosinck, 2011,) and some examples have already been described when the hosts have been subjected to abiotic stresses. For instance, *Cucumber mosaic virus* (CMV) has been observed to confer drought tolerance when the virus is inoculated in different hosts (Xu *et al*., 2008). Some plant viruses such as those causing mild symptoms have also been used for cross-protection against more severe virus strains causing acute disease symptoms (Fraser, 1998). Other beneficial and mutualistic interactions have been described involving plant responses to nutritional needs or stress conditions (Roosinck, 2011, Stobbe and Roosinck, 2014).

Black grass (*Alopecurus myosuroides*) is an annual grass-weed associated with serious yield losses. It is mainly found in the cereal growing areas causing severe crop yield reduction through competition for nutrients, especially nitrogen, within a field. The use of herbicides is the primary method of control and many of them have resulted in satisfactory results for decades. However, in recent years, many of the black grass populations have evolved resistance in many countries in the North of Europe, including the UK where it has been described in more than 35 counties (Hull *et al*., 2014). The development of resistance has resulted in increased interest in the study of alternative control measures or the development of herbicides with new modes of action for which no resistance has been developed.

Currently, two main types of herbicide resistance have been described in black

grass: The first one is known as target-site resistance (TSR), which is characterized by the presence of point mutations in genes encoding proteins involved in the response to the herbicides (Marshall *et al*., 2013; Moss *et al*., 2007). The most important mutations described are in acetolactate (ALS) and acetyl CoA carboxylase (ACCase) proteins. This type of resistance is usually controlled by the use of alternate herbicides with different modes of action. Non-targeted-site resistance (NTSR) is the most widespread and problematic type of resistance and it was first described at Peldon in Essex, England, in 1982 (Heap, 2014). It is driven by inheritable differences in the expression of several genes involved in xenobiotic detoxification and influenced by environmental factors. The study of this type of Multiple-herbicide resistance (MHR) resulted in the identification of a glutathione transferase, termed *Am*GSTF1, which plays a key role in the detoxification of xenobiotics (exome) (Cummins *et al*., 1999). When the *Am*GSTF1 was expressed in transgenic *Arabidopsis thaliana* plants, resistance to different herbicides was observed as previously seen for MHR in weeds (Cummins *et al*., 2013). It was also observed that in black grass populations displaying NTSR, the levels of expression of this protein were higher.

In recent years there has been an increasing interest in the study of the whole microbial community associated with plants. Previous results have indicated that endophytic microbes might influence the development of tolerance to herbicides (Edwards, 2015). Undescribed viruses were found in a Next Generation Sequencing (NGS) study investigating the natural microbiome in black grass. These novel viruses were called Alopecurus myosuroides partitivirus 1 (AMPV1) and Alopecurus myosuroides partitivirus 2 (AMPV2), both of them within the *Partitiviridae* family, and Alopecurus myosuroides varicosavirus 1 (AMVV1) in the *Varicosavirus* genus. No relation has been found between the presence of these viruses and the development of disease symptoms so far (Sabaddin *et al*., 2016). All the viruses have a bi-partite genome structure and two pairs of primers were designed for their detection using RT-PCR to confirm their presence in the infected plants.

Surprisingly, these viruses were found to be more abundant in populations displaying NTSR herbicide resistance compared to wild-type sensitive (WTS) populations.

The discovery of these viruses led to the hypothesis that they could be related in some way with the development or enhancement of resistance, along with the expression of *Am*GST1. In this chapter different black grass populations were assessed to establish the incidence of the new viruses. Also, using a NTSR population from Peldon (Essex), a study between the presence of the viruses and the expression of *Am*GSTF1 was carried out.

6.2 Materials and methods

6.2.1 Plant material

Seeds from different herbicide-susceptible and resistant populations were sown and grown in the glasshouse at FERA under controlled conditions (12:12 h photoperiod and average temperature of 18-20 °C). Populations consisted of black grass samples, collected from 2002 onwards in different regions around the UK and previously classified as WTS, TSR or NTSR. Plants from the Peldon population (31) were grown under the same conditions for the study of the expression of *Am*GSTF1.

6.2.2 Nucleic acid extraction and RT-PCR setup

Total RNA from individual plants from six different populations were extracted using the Kingfisher® mL system (Thermo labsystems) following the method described in Mumford *et al.,* (2004). Samples were eluted in 200 µl of molecular grade water and stored at -20 °C. The samples were quality checked using the Cytochrome oxidase assay (COX), then all the extracts were tested using primers targeting the coat protein region of the RNA2 of AMVV1, AMPV1 and AMPV2. Total RNA (1 µl) was added to a 24 µl reaction from the Verso™ 1-Step RT-PCR ReddyMix™ Kit (Thermo Scientific) and 400 µM of each primer. The assays were performed in a Bio-Rad C1000™ thermal cycler (Bio-Rad laboratories) and cycling conditions consisted of 48 °C for 30 mins for the reverse transcription step, 98 °C for 2 mins, followed by 35 cycles of 98 °C for 10 s, 63 °C for 30 s and 72 °C for 1 min and a final step of 72 °C for 5 min. RT-PCR amplification products were then separated on a 1.2 % agarose gel stained with ethidium bromide (120 V) and visualised in a UV transilluminator.

Table 6.1: Reverse transcriptase-PCR primers used for the RNA2 region of the black grass viruses, AMPV1, AMPV2 and AMVV1.

	Primer	Sequence (5'-3')
AMPV1	**AMPV1-CP F**	ACGCCACTGAACAATTCACTGGCTC
	AMPV1-CP R	TTGAGCCGACGAAGAAGCGACTGTAC
AMPV2	**AMPV2-CP F**	TCACCCGCTTTGGATACTATTGGGTTGC
	AMPV2-CP R	ATCAAAGCCTATGATGGGGCTCTGTGACTCTAG
AMVV1	**AMVV1-CP F**	TACCTGACTCTGACAACTCAAAGGAGCCAGG
	AMVV1-CP R	TAGTCCTGTGCCAGAGTCCACTGCTTAGTTC

6.2.3 Protein extraction and quantification

Leaf samples (31) from the Peldon population were weighed, ground up under liquid nitrogen in a mortar and then 10 x (W:V) extraction buffer consisting of 50 mM Tri-HCl, 2 mM EDTA and 1 mM DTT pH 7.5, and PVPP (50 g/L) was added. The homogenate was mixed and filtered through two layers of miracloth (Calbiochem) to obtain a clarified crude extract, and subsequently centrifuged for 15 min at 10000 g at 4 °C. The supernatant was transferred to a clean tube and the amount of protein was calculated using the Pierce BCA Protein Assay kit (Thermo Scientific) using the Ultraspec 3300 pro Spectrophotometer following the manufacturer's instructions.

6.2.4 SDS-PAGE

SDS-PAGE was performed utilizing 12.5 % acrylamide gels. Each sample (10 µl) and ladder (PageRuler plus Prestained Protein Ladder, Thermo Scientific) were loaded into the wells and the gel was run at a fixed voltage of 100 V in 5X SDS-PAGE running buffer

(125 mM Tris, 0.96 M Glycine and 0.5 % (w/v) SDS pH 8.3) until the dye front exited the bottom of gel. Resolving buffer for gel preparation consisted of 1.5 M Tris-HCl pH 9.0 with 0.4 TEMED (N, N, N', N'-tetramethylethylenediamine) and 0.4 % SDS (sodium dodecyl sulphate) and stacking buffer consisted of 0.14 M Tri-HCl pH 6.8, 0.11 % TEMED and 0.11 % SDS.

6.2.5 Western Blot

Western blotting was performed following SDS- PAGE on 12.5 % acrylamide gels using a PVDF (polyvinylidene difluoride) membrane. All the materials used for the western-blot including the PVDF membrane were soaked in transfer buffer (0.2 M Glycine, 25 mM Tris, 50 ml ethanol) and western blotting was performed for 1 h at a fixed current of 350 A in the Western-blotting tank (Bio-Rad). The membrane was incubated with Tris buffer saline (TBS) buffer and 3 % milk (blocking buffer) for 1 h to prevent nonspecific binding, washed with TBS and incubated for 1 h in dilution buffer (TBS + 1 % milk) with 1/500 *Am*GSTF1 dilution. After washing, anti-rabbit IgG with horseradish peroxidase activity (HRP) was used as a secondary antibody in 1/1000 dilution. Amersham™ ECL™ Western Blotting Detection Reagents kit (GE Healthcare) was used for subsequent substrate chemiluminescent detection. Visualisation of the Western-blot products was performed using a photographic film. MHR positive and WTS negative controls used were provided by Professor Edwards's team from the University of Newcastle.

6.3 Results

6.3.1 Abundance of new viruses in different black grass populations

Amplification products after gel electrophoresis of the extracted samples resulted in products of 728 bp, 248 bp and 986 bp for AMPV1, AMPV2 and AMVV1 respectively (Figure 6.1). In all cases, the assays were performed using primer pairs targeting the coat protein region in RNA2 of each virus. No amplification products were observed in the healthy and negative controls consisting of RNA extracted from a healthy plant and nuclease free water. Positive results for each population are summarized in Table 6.2. Some viruses were seen to be more abundant in certain populations, but at least one of the viruses was found to be present in all of them. Peldon 05 and Peldon 07 were the populations with higher numbers of infected plants and presence of all the viruses, including AMVV1. However, results from Roth 09 population, characterized as WTS and commonly used as a negative control, resulted in two positive plants for AMPV2. Two plants from Notts 05 population, known to present TSR resistance, also tested positive for both Partitiviruses, AMPV1 and AMPV2

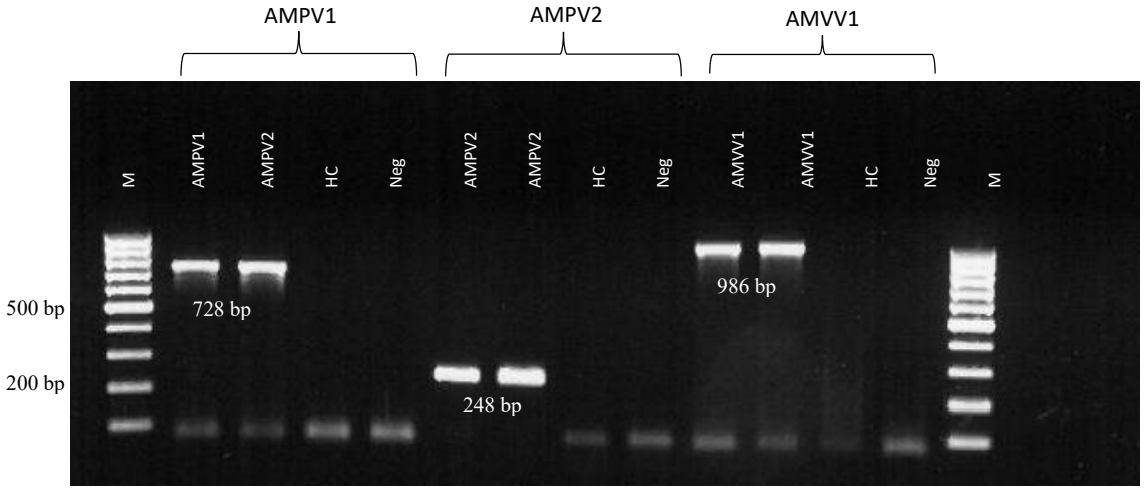


Figure 6.1: RT-PCR products obtained for AMPV1, AMPV2 and AMVV1 respectively. Lanes 1 and 2 are virus positive samples, lane 3 corresponds to the healthy control and lane 4 is the negative control consisting of water,

Table 6.2: Results from the RT-PCR testing using different black grass populations.

Population	Numbers of infected plants/plants tested		
	AMVV1	AMPV1	AMPV2
Peldon 05	21/36	23/36	16/36
Cambridgeshire 08	0/5	4/5	3/5
Peldon 07	5/5	4/5	3/5
Notts 05	0/5	2/5	2/5
Roth 09	0/5	0/5	2/5
Kent1 02	0/5	2/5	1/5

***WTS (wild-type sensitive):** Roth 09; **TSR (target-site resistance)**: Notts 05, Cambridgeshire 08, Kent1 02; **NTSR (non-targeted site resistance)**: Peldon 05, Peldon 07.

6.3.2 Study of the relationship between the presence of new viruses and *Am*GSTF1 expression

A total of 31 black grass plants from the Peldon 05 population were analysed for the expression of the protein *Am*GSTF1 by Western blotting after SDS-PAGE. Prior to Western blot analysis, plants were tested for the presence of the viruses using RT-PCR and classified in different groups from I to VI according to virus infection (Table 6.3). No significant phenotypic differences were seen between groups when plants were visually inspected.

Results from the Western blot are presented in Figure 6.2. Samples were analysed in groups of ten plants each time. The two last wells of each figure correspond to the positive (MHR) and negative (WTS) controls respectively. Results indicated different levels of expression of *Am*GSTF1; some samples (e.g. 2, 4, 13, 14, 27, 28) gave a more intense band than others (5, 8, 18, 21, 22) but no correlation was found between the

expression of the protein and virus infection or group classification. MHR positive control resulted also in a higher expression of the *Am*GSTF1 when compared to WTS control.

Table 6.3: Group classification carried out according to virus infection

Group	Virus infection	Plant numbers
I	AMVV1	10, 14
II	AMVV1, AMPV1	8, 23, 28
III	AMVV1, AMPV2	4, 7
IV	AMVV1, AMPV1, AMPV2	18, 20, 25
V	-	12, 29
VI	AMPV1, AMPV2	2

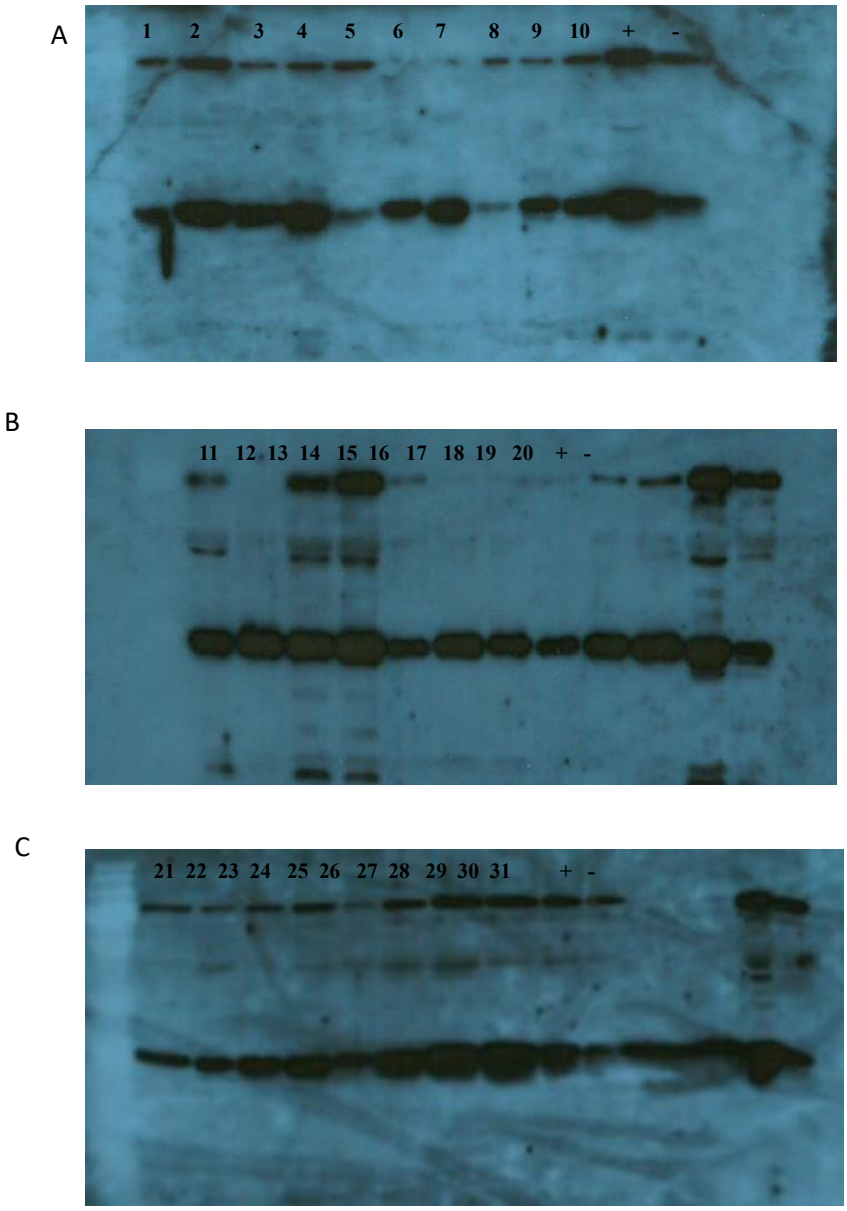


Figure 6.2: Western blot results for the expression of *Am*GSTF1 obtained from 31 black grass plants from Peldon 05 population. (A) samples 1-10, (B) samples 11-20, (C) samples 21-31. Last two lines in each figure correspond to positive (MHR) and negative (WTS) controls respectively.

6.4 Discussion

In recent years there has been an increasing interest in the study of the presence of novel viruses in asymptomatic plants and the interaction with their hosts at the transcriptomic level. Several assays were performed in this study in order to assess the presence of new viruses in different black grass populations and establish their role in the plants. The results obtained would provide a better picture and understanding of the presence of the viruses, which are not associated with the appearance of any disease symptom.

Results from the initial RT-PCR testing indicated the presence of the viruses in all the populations, suggesting that these viruses are more widespread across the UK than initially thought. However, their incidence was notably different among the populations and higher incidence was found in the ones previously characterized as herbicide resistant such as Peldon 05 or Peldon 07. Furthermore, plants from these populations were the only ones which tested positive for AMVV1. These results are in correlation with previous NGS data indicating that the novel viruses were more abundant in populations displaying NTSR resistance than herbicide susceptible populations.

The up-regulation of *Am*GSTF1 protein is known to be strongly correlated with the development of herbicide resistance (Cummins *et al*., 1999, Cummins *et al*., 2013,). Results from the western blot analysis using plants from a NTSR population (Peldon 05) did not indicate any relationship between the expression of *Am*GSTF1 and the presence of the viruses in the plants. AMVV1, AMPV1 or AMPV2 infected plants (groups I, II, III, IV and VI) did not result in a higher expression of *Am*GSTF1 as they did not give a more intense band in the western blot than virus free plants (group V), so no links could be established between these two parameters. However, although no relationship could be found, previous NGS results did indicate the increase titre of the viruses in herbicide resistant plants. The increase titre could suggest that viruses might be somehow correlated

with the enhancement of herbicide resistance through an indirect mechanism involving expression of *Am*GSTF1 or a secondary independent pathway. Examples have already been described following this hypothesis when the hosts are subjected to abiotic stress conditions. For instance, the presence of CMV was seen to reduce the size of its hosts, reducing water requirements and improving water retention when the plants were subjected to drought conditions (Xu *et al*., 2008). Other viruses, even if asymptomatic, have been seen to alter the expression patterns of host genes involved in the synthesis of metabolites, cell cycle, response to other biotic and abiotic stress, photosynthesis, tolerance, and defence mechanisms (Dardick, 2007, Chen *et al*., 2014).Viruses can also alter insect's behaviour; for instance, *Bemisia tabaci* biotype Q was seen to increase its fecundity and longevity periods when feeding from *Tomato yellow leaf curl China virus* (TYLCCNV) infected plants comparing to when feeding from non-infected plants (Liu *et al*., 2010). An alternate hypothesis could be that up-regulation of *Am*GSTF1 could benefit the replication of viruses in the plants. The family of glutathione transferase proteins are usually involved in, not only detoxification of xenobiotics and metabolism, but also in a wide range of cellular processes related with defence, transport of metabolites and cell proliferation (Cummins *et al*., 2011). Replication of the viruses might be benefited under changes of expression of certain genes involved in those mechanisms. The expression of *Am*GSTF1 in transgenic plants and post inoculation of the viruses in transgenic and non-transgenic plants could help us to compare virus titrations in both populations.

In light of the previous results and in order to establish the role of the new viruses in the infected plants in enhancing plant stress tolerance mechanisms, further work is currently being carried out as part of a new PhD at FERA. Infected and uninfected black grass plants will be subjected to different abiotic conditions such as drought, salt or extreme temperatures and plant response will be evaluated in each case.

Chapter 7

General discussion

At the beginning of 2014, the limited knowledge of viral communities in carrot crops in the UK and the previous discovery of novel viruses with unknown impact, led to the development of further studies which helped to gain knowledge of the current situation of the crop. In order to achieve this, it was essential to assess and identify the presence of known and novel pathogens and understand their etiology. Some of the new viruses discovered recently in different hosts have not been related with the development of symptoms. Therefore, possible effects of asymptomatic infection were also investigated.

From the research to industry

The overall losses due to plant pathogens are difficult to quantify as they have environmental, social and economic impact. The first and obvious consequence of viral infection is a reduction in yield and crop production. Prevention is postulated as the first approach to avoid infection and agronomic practices favourable for the production of each crop should always be taken into account and put into practice. Some of these practices might be crop rotation, the use of virus-free seeds or some more advanced methods like breeding for resistance or GM (figure 7.1). Sometimes, individual measures might be inefficient and a combination of them are needed to achieve an effective and long term control (integrated approaches) (Jones *et al*., 2004). The development of new technologies

such as Next Generation Sequencing (NGS) can also be used as a prevention approach as it can identify future threats and new viruses without any previous information.

Confirmation of the discovery of a new virus using NGS requires taxonomic assignment according to the parameters established by the International Committee on Taxonomy of Viruses (ICTV) (King *et al*., 2012), and detection by a complementary detection method (PCR, ELISA, etc). The development of a detection method allows the evaluation of the presence of the new virus in the field, and identification of transmission vectors and possible virus reservoirs. These integrated actions contribute to the evaluation of the impact of a new pathogen within a crop in order to plan a successful management strategy. These strategies are recommended to be specific for each pathogen, robust, economically sustainable and environmentally friendly and the performance and outcome of the previous actions requires a collaborative work between farmers, researchers and industrial companies.

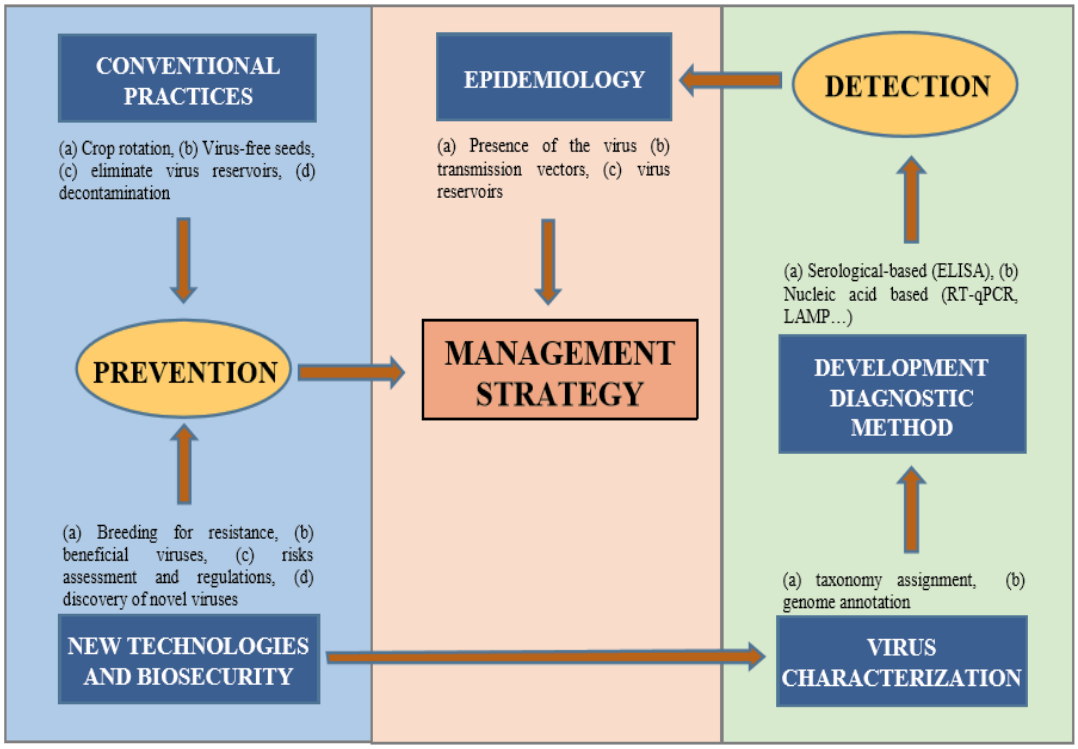


Figure 7.1: Flowchart describing the recommended steps for prevention and early detection of a pathogen in order to set a successful management strategy.

Impact of new viruses within a crop

The first step in the development of a management strategy for any given virus includes the evaluation of its presence within a given ecosystem. The presence of symptoms in the field has always been a starting point to assess virus presence but the recent understanding about the prevalence of asymptomatic viruses makes it necessary to include asymptomatic plants in any survey. Field surveys carried out during this work indicated the high levels of infection in carrot crops across the UK and the elevated presence of the novel viruses Carrot closterovirus 1 (CtCV1) and *Carrot torrado virus 1* (CaTV1). However, the presence of mixed infections with known carrot viruses such as the viruses causing Carrot mottle disease (CMD), which are known to cause disease symptoms, made it more difficult to establish a relationship between the presence of the new viruses in the assessed fields and the development of symptoms and impact in crop production. CaTV1 was also found for first time in a single field carrot crop characterized by year-round cultivation in France

The study of disease epidemics implies the identification of critical points in the disease cycle, identifying factors which contribute to the spread and studying those which might interfere in the interaction between the virus and the host (Jones *et al*., 2004). One aspect is usually the identification of possible virus reservoirs responsible for the source of virus in the field. Morgan *et al*. (2004) suggested cow parsley (*Anthriscus sylvestris*) as a potential virus reservoir for *Parsnip yellow fleck virus* (PYFV). However, it was observed that the virus form in cow parsley/carrot is different to that in hogweed (*Heracleum suphondylium*), which was seen to be similar to that affecting celery (*Apium graveolens*) or parsnip (*Pastinaca sativa*). The reverse was observed in *Carrot red leaf virus* (CtRLV). Analysis of the virus genome sequences from carrot and different weed species indicated they cluster in different clades and suggested that in this case infection in the crop could not have been by transmission from those weed hosts (unpublished results). In this thesis, *Carrot yellow leaf virus* (CYLV), CtCV1 and two new Betaflexiviruses were found to be

present in cow parsley and hogweed samples surrounding carrot crops. However, genome analysis showed few differences between them, though comparing sequences from both hosts was difficult as only short stretches of sequence were generated. Consequently, additional studies are needed to confirm whether any of these two weed species could be potential sources of infection to the crop. Testing of the weed species for *Carrot torrado virus 1* (CaTV1) demonstrated that all the samples were negative for the virus and suggested that infection of the crop was unlikely to be from any of the weed species tested and that other reservoir species might be involved (Fox, 2015). Another possibility might be that the virus is transmitted through seeds.

Identification of the agent responsible for virus transmission can be translated into the application of practices focused to limit and minimize vector activity. CaTV1 transmission studies led to the first demonstration of aphid transmission of a member of the *Torradovirus* genus (Rozado-Aguirre *et al*., 2016). Recently the report of aphid transmission of *Lettuce necrotic leaf curl virus* (LNLCV) in the Netherlands confirmed different transmission mechanisms for non-tomato infecting Torradoviruses (Verbeek *et al*., 2017). Thus control of aphids might limit the spread of these viruses in crops.

Crop rotation has been proposed as a successful strategy to avoid infection for many crops as sometimes, certain pathogens can remain in the soil for a period of time (Abawi *et al*., 2000). However, leaving carrot crops in the ground over winter is widely used in the UK, where high incidence of viruses was found. These results were supported by the ones obtained in France, where higher levels of infection were observed and the presence of CaTV1 was only reported in a year-round cultivation field. Including carrots in rotation with other crops, or at least breaking the growing season leaving the land fallow for a period of time, may be an effective way of controlling the virus in carrot crops.

Finally, Next Generation Sequencing (NGS) was used to study viral communities in carrot crops as an approach for early detection which could lead to the introduction of

control measures preventing future diseases. A novel virus from the *Carlavirus* genus was identified in samples collected in 2015 in the UK. Confirmation of the presence of this new viral sequence in carrots should be carried out using a second detection method such as RT-qPCR and taxonomical assignment should be performed.

Nect Generation Sequencing has been proved as a surveillance tool for field samples diagnosis. Its use has been compared to qPCR, as it allows screening and rapid detection and identification of viruses in a large number of field samples in a single run. However, the cost associated with each run is still very elevated to be used as a routine diagnostic technique. The current development of new, smaller and more economic systems will allow the screening of large batches of samples in short period of times at a reasonable price.

In light of the high numbers of viruses discovered in recent years using NGS, Massart *et al*., (2017) established a progressive scientific guideline for action which describes the steps required for a complete and efficient biological characterization and risk assessment of a new pathogen. It also establishes the need to create appropriate communication channels with the regulatory authorities when a new virus is identified.

Effects of virus infection

The advent of non-targeted diagnostic technologies is resulting in the identification of new viruses which are not necessarily related to disease. The discovery of new viruses has brought into focus the likelihood of a high number of viruses which remain unknown. CaTV1 was a sequencing identified virus not connected with the development of symptoms. Mechanical inoculation experiments in the glasshouse did not result in successful transmission so symptom development could not be assessed. However, transmission of CaTV1 was successful with *Cavariella aegopodii* and *Myzus persicae*, with M. persicae observed to be a more efficient vector. Symptoms developed due to infection consisted in interveinal chlorosis and stunting. However, due to the 'unnatural' growing conditions of the infected chervil plants, the developmet of the symptoms due to infection could not be certified.

In other studies, virus positive black grass plants were not seen to develop any disease symptoms when compared to virus free plants in the glasshouse. However, the greater abundance of virus reads in the NGS data from herbicide resistant plants led to the hypothesis that infection could have some consequences at the transcriptomic level, stimulating or inhibiting the expression of certain genes. It was also proposed that changes in the metabolism of the infected plants might benefit the replication of the viruses (Sabaddin *et al*., 2016). All these previous results could be linked with recent hypothesis and studies, where viruses were proposed as potential agents which might not induce disease (Roosinck 2011a, 2011b). Bacteria, initially also described as pathogenic, have been seen later to play a key role in the development of essential processes in plants and the human body. Although viruses are natural pathogens which need the host machinery to replicate, they could also indirectly produce masked or even desirable changes in their hosts under certain conditions such as external biotic or abiotic stress. However, all these effects have been poorly studied so far and current results are not conclusive. A better knowledge of all these mechanisms involved in virus infection and their consequences could be used

either to mitigate undesirable effects or to take advantage from some beneficial ones and consequently, improve or enhance crop production.

Impact of virus infection and further studies

Field surveys carried out in this study revealed the elevated presence of virus infection within carrot crops, with levels of infection ranging from 80-100 % in some fields, and proposed year-round cultivation as a detrimental practice. Despite this surprising viral load, results do not seem to be translated into the field at first glance. A possible hypothesis is that asymptomatic viruses such as CaTV1 or the Betaflexiviruses could be causing a reduction in yield and crop production and that losses could be unnoticed without the development of obvious visual symptoms. They could also act reducing the size or affecting the taste and quality of the crop. A different approach is that infection could produce beneficial effects in the infected plants under certain conditions. At this point, further characterization studies, development of more economic and quicker screening techniques and large scale surveys comparing infected and non-infected fields would be required in order to evaluate whether the elimination or reduction of these asymptomatic viruses would be translated in any changes in yield and crop production.

Chapter 8

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