Luminex xTAG detection of *Cucumber mosaic virus* (CMV) and different tospoviruses as well as further characterization of CMV with infectious full-length clones and pseudorecombinants

Von der Naturwissenschaftlichen Fakultät der
Gottfried Wilhelm Leibniz Universität Hannover

zur Erlangung des Grades

Doktor der Naturwissenschaften (Dr. rer. nat.)

genehmigte Dissertation
von
M. Sc. Niklas Bald-Blume

geboren am 01.10.1984 in Menden (Sauerland)

2016

Referent: Prof. Dr. rer. hort. Edgar Maiß

Korreferent: Prof. Dr. rer. nat. Thomas Debener

Tag der Promotion: 04.11.2016

Abstract

Plant virus-induced diseases annually cause significant yield and quality losses on crop plants. This is accompanied by severe economic damages and even endangers food security in affected regions. As plant viruses and their vectors are spread worldwide due to global trade of horticultural and agricultural products, reliable detection methods are enormously important for limitation of spreading. Various detection procedures have been developed for plant viruses. However, only one procedure has established itself for routine testing, the enzyme-linked immunosorbent assay (ELISA). As it does not allow the simultaneous detection of several pathogens in one test, a detection method was established on the basis of the Luminex xTAG technology in this work, that theoretically allows the detection and distinction of up to 500 different nucleic acids in one sample. On the one hand this approach enables a general detection of *Cucumber mosaic virus* (CMV) or of viruses of the genus *Tospovirus* and on the other hand it allows the differentiation of the subgroups of CMV and of the *Tospovirus* species *Tomato spotted wilt virus* (TSWV), *Impatiens necrotic spot virus* (INSV), *Capsicum chlorosis virus* (CaCV) and *Watermelon silver mottle virus* (WSMoV), respectively. All examined CMV isolates were constantly detected and could be differentiated more reliably than by a subgroup specific ELISA. Most of the tested *Tospovirus* species were also verified and could be distinguished. This assay can be combined with other tests based on this technology and provides the possibility for a flexible test procedure encompassing the most important pathogens of certain crops. An alternative method for *Tospovirus* detection using a normal RT-PCR and for the distinction of various tospoviruses by restriction fragment length polymorphism (RFLP) analysis was additionally developed.

Plant viruses have to be characterized in detail to curtail their spread and damages on crop plants. Major criteria are transmission pathways, virulence, symptomatology and their determinants as well as the significance of reassortment and recombination for the creation of virulent virus strains. The coat protein genes of 29 CMV isolates were cloned, sequenced and compared by phylogenetic analyses. Infectious full-length clones were created of four CMV isolates comprising all three subgroups of CMV and causing differently severe symptoms on *Nicotiana benthamiana*. Viruses produced from full-length clones corresponded with the original isolates regarding their symptomatology, what qualifies them for further characterizations of CMV. Three of the four CMV isolates and their full-length clones infected *Cucumis sativus* plants inducing similar mosaic symptoms. The nucleotide sequences of the three genomic RNAs of all four isolates were determined. The full-length clones and pseudorecombinants of the four isolates were further investigated. Thereby the determinant for the severe symptoms on *N. benthamiana*, leaf deformations with shoestring leaves, was located on RNA1 for isolate PV-0474. For the other two isolates causing severe symptoms, PV-0506 und PV-0036, it was shown that the determinant is not confined to a single of the three RNAs.

Keywords: Luminex xTAG technology, *Cucumber mosaic virus*, tospoviruses

Zusammenfassung

Durch Pflanzenviren verursachte Krankheiten führen jährlich zu großen Ertragseinbußen und starken Qualitätsminderungen bei Nutzpflanzen. Dies geht mit schweren ökonomischen Schäden und einer Gefährdung der Ernährungssicherheit in betroffenen Gebieten einher. Da sich Pflanzenviren und ihre Überträger durch den globalen Handel mit gartenbaulichen und landwirtschaftlichen Produkten weltweit verbreiten, sind zuverlässige Nachweisverfahren von enormer Bedeutung, um eine Ausbreitung einzuschränken. Es wurden bereits verschiedene Detektionsmethoden für Pflanzenviren entwickelt, allerdings konnte sich in der Praxis bislang nur ein Verfahren, der *Enzyme Linked Immunosorbent Assay* (ELISA), für Routineuntersuchungen durchsetzen. Da dieser jedoch nicht den gleichzeitigen Nachweis verschiedener Krankheitserreger ermöglicht, wurde im Rahmen dieser Arbeit ein Nachweisverfahren auf Basis der Luminex xTAG Technologie etabliert, mit welcher sich theoretisch bis zu 500 verschiedene Nukleinsäuren gleichzeitig in einem Test detektieren und unterscheiden lassen. Das hier entwickelte Verfahren ermöglicht zum einen den generellen Nachweis von *Cucumber mosaic virus* (CMV) oder von Viren des Genus *Tospovirus*, zum anderen erlaubt es die Unterscheidung der Untergruppen von CMV bzw. die Differenzierung der *Tospovirus*-Arten *Tomato spotted wilt virus* (TSWV), *Impatiens necrotic spot virus* (INSV), *Capsicum chlorosis virus* (CaCV) und *Watermelon silver mottle virus* (WSMoV). Es wurden alle untersuchten CMV-Isolate sicher nachgewiesen und konnten zuverlässiger als durch einen untergruppenspezifischen ELISA differenziert werden. Die meisten getesteten *Tospovirus*-Arten wurden ebenfalls detektiert und konnten eindeutig unterschieden werden. Dieser Assay ist mit weiteren auf dieser Technologie basierenden Tests kombinierbar und bietet so die Möglichkeit für ein flexibles Testverfahren, das die wichtigsten Krankheitserreger von bestimmten Kulturen umfasst. Ein Alternativverfahren zum *Tospovirus*-Nachweis mittels normaler RT-PCR und zur Unterscheidung verschiedener Tospoviren durch Restriktionsfragmentlängenpolymorphismus (RFLP)-Analyse wurde zusätzlich entwickelt.

Um die Ausbreitung von Pflanzenviren und durch sie hervorgerufene Schäden an Kulturpflanzen einzuschränken, müssen sie detailliert charakterisiert werden. Wichtige Kriterien sind dabei Übertragungswege, Virulenz, Symptomatologie und ihre Determinanten sowie die Bedeutung von Reassortment und Rekombination für die Erzeugung virulenter Virusstämme. Von 29 CMV-Isolaten wurden die Hüllprotein-Gene kloniert, sequenziert und mittels phylogenetischen Analysen verglichen. Von vier CMV-Isolaten, die zu allen drei Untergruppen von CMV gehören und unterschiedlich starke Symptome auf *Nicotiana benthamiana* auslösen, wurden infektiöse Volllängenklone erstellt. Hinsichtlich ihrer Symptomatologie stimmten die hieraus erhaltenen Viren mit den Ausgangsisolaten überein, so dass sich die Volllängenklone für weitere Charakterisierungen des CMV eignen. Drei der vier CMV-Isolate und ihre Volllängenklone infizierten *Cucumis sativus* Pflanzen und verursachten ähnliche Mosaiksymptome. Die Nukleotidsequenzen der drei genomischen RNAs wurden von allen vier Volllängenklonen ermittelt. Die Volllängenklone und Pseudorekombinanten der vier Isolate wurden näher untersucht. Hierbei konnte für das Isolat PV-0474 die Determinante für die schweren Symptome auf *N. benthamiana*, Blattdeformationen mit *shoestring leaves*, auf RNA1 lokalisiert werden. Bei den beiden anderen Isolaten mit schweren Symptomen, PV-0506 und PV-0036, konnte gezeigt werden, dass die Determinante nicht auf eine einzelne der drei RNAs beschränkt ist.

Schlagworte: Luminex xTAG Technologie, *Cucumber mosaic virus*, Tospoviren

Table of Contents

1 General Introduction

1.1 Plant viruses

Plant viruses are obligate intracellular parasites and depend on the molecular machinery of the host for their replication. They are found in wild plants as well as in crops and can have very broad host ranges of up to several hundred plant species or can be restricted to a single host species. On crop plants they cause serious damage leading to potentially significant yield losses (Edwards et al., 2001). This has of course a direct economic impact on the producers and can additionally affect food security in struck geographic regions (Legg, 1999). In this work the focus lies on the important group of tospoviruses which affect many plant species and are increasingly distributed as well as on the relevant *Cucumber mosaic virus* which is already spread worldwide and also infects a variety of plant species.

1.1.1 *Cucumber mosaic virus*

Cucumber mosaic virus (CMV) belongs to the genus *Cucumovirus* in the family *Bromoviridae* (Bujarski et al., 2012). More than 1,000 plant species from 85 families including many crops like cucumber, tomato, pepper and melon are affected by this virus all over the world. This is one of the widest host ranges of plant viruses (Jacquemond, 2012; Roossinck, 2001). Severe symptoms can be caused leading to loss of quality and yield. These facts have put CMV on the top ten list of economically important plant viruses (Rybicki, 2015; Scholthof et al., 2011). The virus is transmitted by more than 80 species of aphids in a nonpersistent manner (Gallitelli, 2000). Transmission also occurs mechanically, via the plant parasitic dodder (*Cuscuta* spp.) (Schmelzer, 1957) and with varying success through seeds (Palukaitis et al., 1992; Yang et al., 1997). The genome consists of three essential plus-sense single-stranded RNAs, denominated RNA1, RNA2 and RNA3. RNA1 is composed of about 3,400 nucleotides, RNA2 of approximately 3,000 nucleotides and RNA3 of roughly 2,200 nucleotides (Gould and Symons, 1982; Rezaian et al., 1984; Rezaian et al., 1985). All three RNAs have a 5’ cap while the 3’ ends exhibit a tRNA-like structure (Ahlquist et al., 1981; Symons, 1975). Additionally, two major subgenomic RNAs (RNA4 and RNA4A) are produced (Ding et al., 1994; Schwinghamer and Symons, 1977). The five open reading frames (ORFs) encoding the five viral proteins are distributed over the three genomic and two subgenomic RNAs. The 110 kDa 1a protein with a putative methyltransferase and helicase domain is translated from the monocistronic RNA1 (Habili and Symons, 1989; Rozanov et al., 1992). RNA2 encodes the 98 kDa 2a protein, the RNA-dependent RNA polymerase (RdRp), and the 15 kDa 2b protein on the subgenomic RNA4A, which is a suppressor of gene silencing and plays a role in virus transport (Guo and Ding, 2002; Hayes and Buck, 1990). RNA3 holds the genetic information for the 30 kDa 3a protein, which is the movement protein (MP), and for the 25 kDa 3b protein, which is the coat protein (CP) translated from the subgenomic RNA4 (Ding et al., 1995; van Regenmortel et al., 1972). Both the MP and the CP are necessary for virus movement (Suzuki et al., 1991), while the CP alone is sufficient for aphid transmission (Chen and Francki, 1990). The three genomic RNAs are compartmentalized in three icosahedral particles (Peden and Symons, 1973) with a diameter of 30 nm consisting of 180 copies of the CP (Finch et al., 1967). Sometimes particles also contain subgenomic RNA additional to genomic RNA (Lot and Kaper, 1976; Peden and Symons, 1973). Based on serology, nucleic acid hybridization and peptide mapping of the CP, CMV can be differentiated into the two subgroups I and II (Devergne and Cardin, 1975; Edwards and Gonsalves, 1983; Owen and Palukaitis, 1988; Palukaitis et al., 1992). Further subdivision of subgroup I into subgroups IA and IB can be

achieved on the basis of nucleic acid sequences (Roossinck et al., 1999). The two subgroups I and II cause symptoms of varying severity and differ in virulence. In general subgroup I strains and isolates induce stronger symptoms and are more virulent (Carrère et al., 1999; Wang et al., 2002). In this regard there are no differences between subgroups IA and IB, but they have different geographic distributions with subgroup IB isolates being mainly found in Asia (Roossinck, 2002; Lin et al., 2003). Reassortments and recombinations of RNAs occur between the different subgroups and also between *Cucumovirus* species (Nouri et al., 2014; Chen et al., 2007; Bonnet et al., 2005; Roossinck, 2002; Carrère et al., 1999; White et al., 1995). Symptomatology varies depending on environmental conditions, host species and genotype, virus subgroup, strain and isolate and can range from no symptoms at all to complete systemic necrosis even leading to plant death (Palukaitis et al., 1992; Jacquemond, 2012). Further symptoms include systemic mosaic symptoms, leaf deformation, stunted growth and chlorosis. Some CMV strains can contain satellite RNAs that either aggravate or ameliorate the symptoms caused by their helper CMV strain alone (Kaper and Tousignant, 1984; Sleat et al., 1994; Piazzolla et al., 1998).

1.1.2 Tospoviruses

The genus *Tospovirus* contains all the plant viruses of the family *Bunyaviridae*. Its name is derived from the type species *Tomato spotted wilt virus* (TSWV). The genus comprises the 11 approved and 20 tentative species listed in Table 1.1 (Hassani-Mehraban et al., 2016; International Committee on Taxonomy of Viruses, 2015; Plyusnin et al., 2012).

Table 1.1 The approved and tentative species belonging to the genus *Tospovirus*.

Approved species	**Tentative species**
Tomato spotted wilt virus (TSWV)	Alstroemeria necrotic streak virus (ANSV)
Groundnut bud necrosis virus (GBNV)	Alstroemeria yellow spot virus (AYSV)
Groundnut ringspot virus (GRSV)	Bean necrotic mosaic virus (BNMV or BeNMV)
Groundnut yellow spot virus (GYSV)	Capsicum chlorosis virus (CaCV)
Impatiens necrotic spot virus (INSV)	Calla lily chlorotic spot virus (CCSV)
Iris yellow spot virus (IYSV)	Chrysanthemum stem necrosis virus (CSNV)
Polygonum ringspot virus (PolRSV)	Groundnut chlorotic fan-spot virus (GCFSV) also known as Peanut chlorotic fan-spot virus (PCFV)
Tomato chlorotic spot virus (TCSV)	Hippeastrum chlorotic ringspot virus (HCRV)
Watermelon bud necrosis virus (WBNV)	Lisianthus necrotic ringspot virus (LNRV)
Watermelon silver mottle virus (WSMoV)	Melon severe mosaic virus (MSMV)
Zucchini lethal chlorosis virus (ZLCV)	Melon yellow spot virus (MYSV) also known as Physalis severe mottle virus (PhySMV)
	Mulberry vein banding-associated virus (MVBaV)
	Pepper chlorotic spot virus (PCSV)
	Pepper necrotic spot virus (PNSV)
	Soybean vein necrosis-associated virus (SVNaV) also known as Soybean vein necrosis virus (SVNV)
	Tomato necrosis virus (TNeV)
	Tomato necrotic ringspot virus (TNRV)
	Tomato necrotic spot virus (TNSV)
	Tomato yellow ring virus (TYRV)
	Tomato zonate spot virus (TZSV)

All approved and tentative tospoviruses have a common spherical structure with a diameter of 80-120 nm enveloped by a host-derived membrane. The two viral glycoproteins Gn and Gc are embedded into this membrane and are responsible for virus transmission. Particles contain a tripartite single-stranded RNA genome with negative- or ambisense polarity. The three RNAs differ in size and are thus

called large (L), medium (M) and small (S) segment. All three segments are incorporated in one particle but are independently packaged by many copies of the nucleoprotein (N protein) and a few copies of the viral RNA-dependent RNA polymerase (RdRp) (Kormelink et al., 2011; Turina et al., 2012). The L segment is a negative-sense RNA, which encodes only the RdRp (Adkins et al., 1995). The M segment has ambisense polarity and encodes the precursor polyprotein for the glycoproteins Gn and Gc (Kormelink et al., 1992a) and the NSm protein, which is the movement protein (Lewandowski and Adkins, 2005). The S segment is also ambisense and encodes the NSs protein, a suppressor of gene silencing (Takeda et al., 2002), and the N protein. The four proteins of the M and S segments are expressed through subgenomic RNAs via cap snatching (Kormelink et al., 1992b). Tospoviruses are transmitted by thrips (insect order: *Thysanoptera*) in a persistent and propagative manner, which means that viruses replicate in the insect, circulate through the body and persist over the various developmental stages. In total fifteen thrips species are vectors of tospoviruses and are essential for their epidemiology (Pappu et al., 2009; Rotenberg et al., 2015). Only thrips that acquire the virus as larvae are able to transmit it and the first instar larvae are most efficient in acquiring the virus, while adults are the primary transmitters (Rotenberg et al., 2015). Transmission also occurs mechanically. Seed transmission was generally described not to occur for tospoviruses, but was detected at a low rate for Soybean vein necrosis virus (SVNV) (Groves et al., 2016). General symptoms caused by tospoviruses include ringspots, stunting, chlorosis and necrosis, which vary according to virus and host species (Pappu et al., 2009). These are the common characteristics of all tospoviruses. Concerning their host range and distribution the individual *Tospovirus* species differ significantly from each other. However, host ranges and geographical distributions have increased in recent years for the established *Tospovirus* species. Additionally, new species belonging to this genus have been described.

TSWV was the first described *Tospovirus* reported from Australia around 1920 (Samuel et al., 1930). Since then it has spread worldwide. The host range is similar to CMV with 1,090 plant species in 85 families containing many economically important crop plants like tomato, potato, groundnut, gerbera and begonia as well as numerous weed species (Parrella et al., 2003). It is transmitted by the nine thrips species *Frankliniella occidentalis, F. fusca, F. schultzei, F. intonsa, F. bispinosa, F. cephalica, F. gemina, Thrips setosus and T. tabaci* (Rotenberg et al., 2015). *F. occidentalis* has a worldwide distribution (Kirk and Terry, 2003), while *Thrips setosus* is restricted to Japan (Murai, 2001). Because of the global distribution of TSWV and its vectors, its wide host range and the provoked damages to crops, this virus is considered as one of the most important plant viruses (Rybicki, 2015; Scholthof et al., 2011). *Impatiens necrotic spot virus* (INSV) was first isolated from *Impatiens* sp. (Law and Moyer, 1990). It affects ornamentals like New Guinea impatiens, vegetables like sweet pepper, cucumber and lettuce and weeds like chickweed (*Stellaria media*) (Turina et al., 2012). This virus is distributed worldwide (Pappu et al., 2009) and transmitted by the three thrips species *F. occidentalis*, *F. fusca* and *F. intonsa* (Rotenberg et al., 2015). *Watermelon silver mottle virus* (WSMoV) was first found infecting watermelon in Taiwan (Yeh et al., 1992) and is still restricted geographically to Asia (Pappu et al., 2009). The host range is limited to members of the family *Cucurbitaceae*, which includes the crops cucumber, melon and watermelon. It is transmitted only by *Thrips palmi* (Rotenberg et al., 2015). *Iris yellow spot virus* (IYSV) was first reported on onion from Brazil and has since been found around the world on vegetable and ornamental bulb crops (Bag et al., 2015; Pappu et al., 2009). It is transmitted by *F. fusca* and *T. tabaci* (Rotenberg et al., 2015). *Groundnut ringspot virus* (GRSV) was first isolated from groundnut in South Africa (Avila et al., 1993). It has been found to affect groundnut, soybean,

tomato and sweet pepper production in Africa and South America (Pappu et al., 2009). Recently, it has been detected for the first time in North America on tomato, sweet pepper and weeds (Webster et al., 2015). The thrips species *F. occidentalis*, *F. schultzei* and *F. gemina* are vectors of this virus (Rotenberg et al., 2015). *Tomato chlorotic spot virus* (TCSV) was first found on tomato in Brazil (Avila et al., 1993) and was limited to South America for a long time (Pappu et al., 2009). Only recently, it has been detected for the first time on tomato, sweet pepper and weeds in North America, where it is limited to Florida so far (Webster et al., 2015). This virus is transmitted by *F. occidentalis*, *F. schultzei* and *F. intonsa* (Rotenberg et al., 2015). Capsicum chlorosis virus (CaCV) was first reported on sweet pepper, chili and tomato from Australia (McMicheal et al., 2002) and is restricted to Asia and Australasia until now (Pappu et al., 2009). In addition to solanaceous crops it also infects peanut (Chen et al., 2007). The three thrips species *Ceratothripoides claratris*, *T. palmi* and *F. schultzei* are transmitters of this virus (Rotenberg et al., 2015). Alstroemeria necrotic streak virus (ANSV) was first isolated from *Alstroemeria* sp. in Colombia (Hassani-Mehraban et al., 2010). So far it has not been reported from other countries. *F. occidentalis* can transmit this virus (Rotenberg et al., 2015).

1.2 Detection methods

Plant viruses and their vector species are further distributed worldwide with increasing global trade of agricultural and horticultural products and affect the same plant species in new geographic areas and sometimes also new plant species. This illustrates the importance of reliable detection methods for plant viruses. Plant health services need to ensure that plant material for import and export is virus free or at least free of critical viruses to prevent global spread (quarantine). The European and Mediterranean Plant Protection Organization (EPPO) was founded amongst other reasons to develop strategies and to coordinate efforts of national plant health services against the introduction and spread of dangerous pests and pathogens in the European and Mediterranean region. It publishes the updated A1 and A2 lists of pests and pathogens recommended for regulation as quarantine pests every year. Pests and pathogens on A1 are not present in the EPPO region while those on A2 are already found in some member states. A1 currently lists 24 plant viruses with the two tospoviruses WSMoV and Chrysanthemum stem necrosis virus (CSNV) and A2 specifies 22 viruses with the two tospoviruses INSV and TSWV (EPPO, 2015). Plant health services are also assigned to certify seeds, tubers, young plants, rootstocks and scions for regional cultivation, which should be free of critical viruses to prevent spread into the environment. Additionally, their duty is to identify already occurring viral diseases in the field to contain them. Breeding companies need detection methods to assess the virus resistances of new cultivars. For these purposes the methods need to be reproducible, robust, easy to handle, cost effective and scalable to large sample numbers. Finally, new unknown viruses need to be characterized by suitable methods.

1.2.1 Traditional methods

Traditionally, the description and recognition of virus symptoms on various hosts especially on indicator plants was applied for virus identification (Boonham et al., 2014). This required specialists with years of experience and was still inaccurate, because the symptoms caused by a virus on a certain host plant can vary significantly with environmental conditions and the virus strain or even isolate. In the 1930s the transmission electron microscopy was invented and later applied in plant virology. With this technology, virus particles can be visualized, which allows to narrow down a virus sample to a certain virus group (most likely the family) but not to the exact virus species. Additionally, this method requires

expensive equipment as well as experienced personnel in handling of the microscope and in virus purification. This limited the adoption of this technique to some specialized laboratories.

1.2.2 Enzyme-linked immunosorbent assay (ELISA)

The enzyme-linked immunosorbent assay (ELISA) fulfills the requirements of robustness, reproducibility, cost effectiveness, scalability and simplicity and has developed into the standard method for routine virus testing since its development (Voller et al., 1974) and first applications on plant viruses (Voller et al., 1976; Clark and Adams, 1977). Different assay formats have been developed from this like direct, indirect, plate trapped, double antibody sandwich (DAS) and triple antibody sandwich (TAS) ELISA. Nevertheless, the basic principle is the same for all formats. Virus material is directly bound to a plate or captured by a first antibody coating the plate. Then another antibody specifically interacts with the bound virus material. This can be either directly enzyme-linked or another secondary enzyme-linked antibody is used, which recognizes the primary antibody. Different enzymes can be tied to the antibodies, the most frequently used are the horseradish peroxidase (HRP) or the alkaline phosphatase (AP) which catabolize a substrate leading to a color change or chemiluminescence which can be evaluated visually or by spectrophotometry (Torrance and Jones, 1981). Polyclonal and monoclonal antibodies differing in sensitivity and specificity can both be used for virus detection. Monoclonal antibodies are generally more sensitive and specific and can be produced in greater quantities by immortal hybrid cell lines called hybridomas (Köhler and Milstein, 1975; Dietzgen and Sander, 1982). Different protocols for the varying assay formats, types of antibodies and coupled enzymes have been established and can be adapted for new viruses. All these protocols are easy to implement and use. Results are obtained relatively quickly, are comparable and easy to interpret. So the ELISA has developed into the most widely used method for plant virus detection.

Nevertheless, this method also has its disadvantages. High-quality antisera are required which again needs expertise in the purification of either virions or viral proteins. This is a long and costly development process and the relatively small market for antibodies against plant viruses might not repay the costs. Antisera sometimes lack the specificity to distinguish virus strains that differ in their symptomatology. Some virus strains induce more severe symptoms and it would be desirable to distinguish them from less severe strains like the tuber necrosis strain of *Potato virus Y* (PVY^{NTN}) which is indistinguishable by ELISA from PVY^{N} causing no or only mild symptoms on potatoes (van der Heuvel et al., 1994). A laboratory with some basic equipment is necessary to perform ELISAs, so on-field testing is not possible with this method. Additionally, it cannot be executed in a multiplex format detecting several pathogens simultaneously in the same test. This is a big disadvantage when certain crop samples need to be tested for a group of pathogens like seed potatoes that are screened for different viroids, viruses, bacteria and fungi. A test has to be conducted for every pathogen, which is time-, labor- and cost-intensive.

1.2.3 Lateral flow tests

A solution for on-field detection of plant pathogens are lateral flow tests (LFT), also called lateral flow devices (LFD), assays (LFA), strips (LFS) or biosensors (LFB). They are mainly immuno-chromatographic assays. Most home pregnancy tests use this technique for the detection of the urinary human chorionic gonadotropin (hCG), a hormone produced by the embryo after implantation. The first test was developed for detection of hCG in blood (Hussa and Hudson, 1984) and tests were commercialized in 1988. All these devices have the same structure and work according to the same

principle, no matter what the analyte is. They have a strip form and consist of four sections made from different materials: a sample pad consisting of cellulose, a conjugate pad of glass fiber containing a 1st labelled antibody against the analyte, a detection pad of nitrocellulose with a test line with a fixed 2nd analyte specific antibody as well as a control line with a fixed 3rd antibody against the 1st antibody and an absorption pad of cellulose. A sample is added to the sample pad, it will flow into the direction of the absorption pad due to capillary forces, it will pass the conjugate pad, where the analyte interacts with the 1st antibody. The sample solution will move on and take the analyte with bound antibodies and also unbound antibodies with it. It will reach the detection pad where the analyte with bound 1st antibody reacts with the 2nd antibody. This enriches the labelled 1st antibody in a visible line. Unbound 1st antibody will be captured by the 3rd antibody in the control line. If the sample contains the analyte, two lines will appear. While only the control line will be visible if the test worked but no analyte was present in the sample (Quesada-Gonzalez and Merkoci, 2015). Tests based on this principal have soon been used for the detection of plant viruses. Tsuda et al. (1992) applied them for CMV and *Tobacco mosaic virus* (TMV). Tests for *Plum pox virus* (PPV) and *Potato virus X* (PVX) have later been developed amongst others (Byzova et al., 2010; Drygin et al., 2012). The latest application has been for *Soybean mosaic virus* (SMV) (Zhu et al., 2016). Adaptations of this procedure have been developed to increase sensitivity and to improve evaluation of results. Different nanoparticles can be coupled to the antibodies, some of which allow an electrochemical or magnetic analysis of results. Oligonucleotide or peptide aptamers can also be used instead of antibodies. Smartphones can be used for evaluation. Song et al. (2014) developed a limited multiplex format for the detection of three mycotoxins. Quesada-Gonzalez and Merkoci (2015) give an interesting overview over these recent developments.

1.2.4 Polymerase chain reaction (PCR) methods

Advantages of nucleic acid-based methods are their multiplexing potential and their adaptable specificity compared to ELISA. The most well-known method is the polymerase chain reaction (PCR) amplifying certain DNA fragments with the help of more or less specific oligonucleotide primers (Saiki et al., 1988). For RNA viruses a reverse transcription polymerase chain reaction (RT-PCR) is conducted transcribing RNA into cDNA before its amplification (Vunsh et al., 1990). Of course, RNA or DNA extraction depending on the virus type have to precede the PCR or RT-PCR. The specificity of the reaction depends on the primers. Generic primers can be designed that allow the detection of a group of viruses (e. g. a genus) or very specific primers can be developed for the detection of only a certain virus species or even strain. Continuing the example mentioned above PVY^{NTN} could be distinguished from PVY^{N} with this method (Weidemann and Maiss, 1996). Multiplexing is possible by combining sets of primers. In this case it has to be ensured that their annealing temperatures are in the same range, that there are no cross reactions between the different primer sets and that product sizes are distinguishable or that labelled primers are used if fragments are of similar size. These points and the limited number of primer labels restrict the multiplexing capability to a few targets. Concerning simplicity of implementation and handling as well as costs PCR is comparable to ELISA. A lot of procedures have been described for the detection and distinction of different plant viruses, but only a few are routinely used in diagnostic laboratories (Boonham et al., 2014). This rare adoption for routine testing in comparison to ELISA is due to problems with sensitivity, scalability and contaminations that restrain the reproducibility and robustness of the method. Sensitivity can be a problem, because only minor mutations in viruses affecting the primer binding site might inhibit their detection. Scalability to large sample numbers is hindered by gel electrophoresis which is a time consuming process and additionally

a potential source for sample mix-ups. Handling of PCR products for gel electrophoresis can contaminate the laboratory equipment with small amounts of DNA, which can lead to false positive results in new samples (Boonham et al., 2014).

Quantitative or real-time PCR (qPCR) and reverse transcription qPCR (RT-qPCR) solve the problem of scalability and contamination, because the reaction products are sealed and measured in the tubes or microplates and no gel electrophoresis has to take place. Therefore, fluorescent probes or dyes labelling the amplification products and thermal cyclers with the capacity to illuminate each sample and to detect the resulting fluorescence are used. This method was initially developed to determine gene expression by measuring the fluorescence intensity over the amplification period (real-time). A fluorescence threshold is set in the exponential amplification phase. The cycle in which the threshold is exceeded is called the threshold cycle (C_t). The C_t of the sample is compared with the C_t of either a housekeeping gene for relative quantification or a DNA standard for absolute quantification of the target nucleic acid concentration (Heid et al., 1996; Lie and Petropoulos, 1998). The fluorescent signal can also be determined at the end of the amplification (end point) if no quantification is necessary. Because of the mentioned advantages over conventional PCR, the method has been quickly adopted for diagnostics of plant viruses (Mumford et al., 2000). Various assay formats have been developed. The signal is generated either by fluorescent probes like TaqMan or by fluorescent dyes like SYBR green. TaqMan based assays were early approaches, have been well characterized and are thus adopted most frequently (Boonham et al., 2014). They can also be applied for viroid detection for which antibody based methods do not work (Boonham et al., 2004). Multiplexing is possible as several fluorophores and quenchers are available. This potential has been demonstrated by the simultaneous detection of *Citrus tristeza virus* (CTV), *Citrus psorosis virus* (CPsV) and *Citrus leaf blotch virus* (CLBV) (Osman et al., 2015). The (RT-)qPCR is a good alternative to ELISA with the same positive characteristics like robustness, reproducibility, simplicity and scalability furthermore adding multiplexing potential and increased specificity. This is the reason why an increasing number of routine diagnostic laboratories apply this technology. In terms of costs it requires more expensive equipment and chemicals.

1.2.5 DNA microarray

Nucleic acid spot hybridization (NASH) also known as DNA microarray, DNA chip or biochip was developed to investigate messenger RNA (mRNA) accumulation (Schena et al., 1995). Up to several thousand different microscopic spots each consisting of many copies of identical oligonucleotide probes are synthesized or printed on a solid surface made of glass, plastic or silicon (Dufva, 2005). Then fluorescently labelled DNA (transcribed from RNA or amplified from DNA) is added and can hybridize to complementary probes. The samples can be identified from fluorescence signals and their positions. Various formats of this technique have been developed for different purposes including plant virus diagnostics. Although the technique offers high multiplexing potential, specificity and simplicity in its use, it has not been adopted by routine testing laboratories, because it lacks sensitivity, speed and the potential for high throughput testing (Boonham et al., 2007). It requires a laboratory with expensive equipment.

1.2.6 Isothermal amplification methods

Isothermal amplification methods share with PCR the use of DNA polymerases to elongate primers with the difference that no repeated cycling of denaturation, annealing and elongation takes place. The primers bind to the target and are elongated at a constant temperature. For such a reaction simple and

low power instruments are sufficient compared to thermal cyclers necessary for PCR. This has raised interest in isothermal amplification for field applications and for laboratories with poor resources. Separation of DNA double strands is achieved by non-thermal means through helicases in the helicase dependent amplification (HDA) (Vincent et al., 2004) and through recombinase-primer complexes and then DNA polymerases in the recombinase polymerase amplification (RPA) (Piepenburg et al., 2006). The nucleic acid sequence based amplification (NASBA) (Compton, 1991) requires a single denaturation step for double-stranded nucleic acids (dsDNA and dsRNA) to allow primer annealing for the first reaction. These primers contain a T7 RNA polymerase promoter. After that the mixture of RNA polymerase, reverse transcriptase and RNase works at a constant temperature, producing RNA intermediates, which are hydrolysed after being transcribed into DNA. Leone et al. (1997) used this method for detection of *Potato leaf roll virus* (PLRV). NASBA is not truly isothermal but requires a twostep thermal-profile. For HDA such a profile is not necessary, but is recommended for increased sensitivity. Both methods have relatively long reaction times of about 90 min (Boonham et al., 2014). RPA requires agitation after some minutes for optimal amplification and has a problem with non-specific reaction products, because of its low reaction temperature (Boonham et al., 2014). Rolling circle amplification (RCA) uses the strand displacement activity of the Phi29 DNA polymerase to produce multiple connected single-stranded linear copies of the original DNA. It is restricted to circular DNA viruses. The reaction can be combined with a restriction fragment length polymorphism (RFLP) analysis to obtain smaller and specific DNA fragments. Haible et al. (2006) showed that this approach worked well for geminiviruses with a single-stranded circular DNA genome. Loop-mediated isothermal amplification (LAMP) is performed at a constant temperature of 60-65 °C after an initial denaturation. Four to six primers are used for this reaction. Two internal primers are designed to form loops of single-stranded DNA in the reaction products, which are targets for further primer annealing. Another set of external primers is required for the displacement of the internal primer products from the original target DNA utilizing a DNA polymerase with a high strand displacement activity (Notomi et al., 2000). A third pair of loop primers can be added to accelerate the reaction by priming to the loop regions in the orientation in which the internal primers cannot bind. The initial denaturation can also be omitted (Nagamine et al., 2001). For RNA virus detection, reverse transcription of RNA and amplification of cDNA can proceed simultaneously at a temperature of around 60 °C in RT-LAMP (Fukuta et al., 2003). Reaction products are a mixture of stem-loop DNAs and cauliflower-like structures with multiple loops of various lengths. The reaction can be performed in 30 min. An important point for on-site testing is its tolerance of some substances which are inhibitory to PCR simplifying nucleic acid extraction (Kaneko et al., 2007). The amplification is highly efficient and produces large amounts of magnesium pyrophosphate as a by-product. This enrichment results in an increasing turbidity that can be observed with the naked eye or measured photometrically. Addition of metal ion indicators like calcein with manganese chloride ($MnCl_2$) and hydroxynaphthol blue before incubation or of intercalating dyes like SYBR Green after incubation can increase reliability. Products can also be analyzed by gel electrophoresis. All post reaction manipulations are again a source for contaminations of subsequent tests in laboratories. Electronic devices can be used to monitor reactions in real-time measuring turbidity, fluorescence of added intercalating dyes or probes or bioluminescence produced by an added firefly luciferase. Added chemicals and measuring devices increase costs and can prevent on-field applicability. Different variants of this method have been applied for the detection of human pathogens like the human African trypanosomiasis (Wastling et al., 2010) and for plant pathogens like the *Plum pox virus* (PPV)

(Hadersdorfer et al., 2011). All these assays have the disadvantage that they cannot be performed in a multiplex format.

1.2.7 Microsphere-based methods

In routine diagnostics, samples like seed potatoes often have to be tested for various pathogens. To reduce work load, it would be desirable to combine all necessary tests into one assay. Therefore, a multiplex format is of interest. Luminex (Austin, USA) has developed an array platform that allows the simultaneous detection of up to 500 analytes in one sample. The centerpiece of this platform are the 500 different MagPlex Microspheres containing mixtures of two to three fluorophore dyes at different ratios. The polystyrene microspheres, also called beads, have a diameter of 6.5 µm and are magnetic facilitating washing procedures to reduce background signals. They can be covalently coupled with either antibodies, oligonucleotides, proteins, peptides, lipids or polysaccharides. For plant virus detection either antibody or oligonucleotide coated microspheres are suitable. Different virus-specific antibodies can be coupled to differently dyed xMAP beads and these varying microspheres can be combined to create sets for the detection of combinations of plant viruses and other pathogens occurring in certain crops. The test procedure is identical to a DAS-ELISA. Plant samples are prepared with standard ELISA buffers and incubated in standard 96-well micro plates together with the antibody-coated beads. Viruses in the plant samples are captured by the antibodies coupled to the microspheres. After washing, a mixture of secondary antibodies conjugated with the same fluorophore reporter (e.g. Alexa Fluor 532) is added. Samples are incubated and washed again. Afterwards analysis takes places in a flow cytometer or similar instrument. Samples are pumped through a small capillary only fitting one bead in a row passing two lasers or LEDs and corresponding image analyzers. A red laser or LED with a wavelength of 635 nm excites the bead dye mix and, for example, a green laser or LED with a wavelength of 532 nm excites the fluorophore reporter (e.g. Alexa Fluor 532). The resulting fluorescence of the bead dye mix and the reporter molecule, respectively, are measured and analyzed. The signal of the reporter states that virus is present and gives an approximation of the virus titer through the median fluorescence intensity (MFI) value similar to ELISA values. The signal of the bead dye mix allows the identification of the sample. The whole procedure is described in detail by Rao et al. (2004) and only necessitates 3-4 h which is much faster than a typical ELISA taking 1-2 days. It has proven its functionality and multiplexing capability in the detection of human pathogens (Dunbar et al., 2003) and plant viruses (Bergervoet et al., 2008). In a similar approach the nucleic acids of plant viruses can be targeted by the Luminex xTAG technology. Here the already described MagPlex Microspheres are coated with specific oliconucleotides called anti-tags. Each anti-tag correlates with a specific bead dye mix. Viral DNA is amplified by a PCR and viral RNA is first transcribed into cDNA and subsequently amplified (RT-PCR). Different primer sets can be used in this multiplexed pre-amplification step, which enriches the target sequences for the following target specific primer extension (TSPE) reaction. In this linear amplification step biotinylated dCTP is integrated into the extending primers. The TSPE primers have a chimeric layout of a virus-specific part allowing amplification of viral (c)DNA and an oligonucleotide tag, which serves to hybridize the TSPE products to complementary anti-tags on the MagPlex-TAG Microspheres in the next step. This reaction can be multiplexed with primers for different viral targets. The TSPE products are hybridized to the microspheres via a tag to anti-tag interaction. After a washing step the fluorophore reporter protein streptavidin-R-phycoerythrin is added and binds to the incorporated biotin in the TSPE products. Afterwards the bead-TSPE product complexes are analyzed similarly as described above for the xMAP assay. In the instrument a red laser or LED excites the dye mix of individual beads again and

a green laser (532 nm) the bound streptavidin-R-phycoerythrin. The resulting fluorescences of the bead dye mix and the reporter protein are measured and analyzed again. The signal of the reporter states that virus is present, while the signal of the bead dye mix allows the identification of the sample. The procedure is described in detail by van der Vlugt et al. (2015) and takes 1-2 days similar to an ELISA. This technology has also already proven its functionality and multiplexing capability in human medicine for the diagnosis of twenty different respiratory viruses and subtypes (Mahony et al., 2007), of seven gastroenteric viruses (Liu et al., 2011) and of the ten most clinically relevant *Escherichia coli* subtypes (Lin et al., 2011). In recent years it has been applied in phytomedicine for the diagnosis of different pospiviroids (van Brunschot et al., 2014b), begomoviruses and their whitefly vectors (van Brunschot et al., 2014a) and three lily-infecting viruses (Lim et al., 2016). The costs for the necessary equipment are high, but the multiplexing potential can greatly reduce time- and labor-input and thus the costs compared to simplex detection methods for high throughput testing laboratories. Both methods are restricted to laboratory application.

1.2.8 Next-generation sequencing (NGS)

Most of the methods described so far can only detect known pathogens. Additionally, some minor genetic variations can already disturb their functionality. Hence methods for the diagnosis of new pathogens and new strains are needed. Next-generation sequencing (NGS) allows this and has developed rapidly in the last years with ever decreasing costs for equipment and sequencing runs. The term refers to a collection of sequencing technologies that have been developed in the last decade including sequencing by synthesis (Illumina) (Bentley et al., 2008), sequencing by ligation (SOLiD) (Shendure et al., 2005), ion semiconductor sequencing (Ion Torrent) (Rothberg et al., 2011), pyrosequencing (Roche 454) (Margulies et al., 2005) and single-molecule real-time sequencing (Pacific Biosciences) (Eid et al., 2009). All these methods differ in their characteristics like read length, number of reads per run, accuracy of single reads and the time per run connected with variable costs and certain advantages and disadvantages for each method. Illumina sequencing (Kreuze et al., 2009; Wylie and Jones, 2011) and Roche 454 sequencing (Adams et al., 2009; Hagen et al., 2012) have been applied for sequencing of plant viruses. Different ways of nucleic acid preparation for sequencing have been tested. Rolling circle amplification has been used by Hagen et al. (2012) for amplification of geminiviruses before sequencing. Total mRNA was directly transcribed into cDNA and then sequenced by Wylie and Jones (2011). Adams et al. (2009) used subtractive hybridization to enrich non-plant RNA before sequencing. Kreuze et al. (2009) assembled a complete viral genome from purified and sequenced small interfering RNAs (siRNAs). The equipment is still very expensive restricting its operation to specialized laboratories. Some offer sequencing as a service. But this is still connected to high costs. Additionally, sample preparation, shipping of samples, sequencing and data analysis altogether take quite long.

Table 1.2 summarizes the characteristics of the described detection methods.

Table 1.2 Characteristics of the different detection methods. Methods based on amplification of DNA can also be used for RNA detection by including a reverse transcription step (RT).

	On-field	Speed	Costs	Simplicity	Sensitivity	Specificity	Reproducibility	Contamination	Multiplex capability	High-Throughput
Symptomatology	Yes	Slow	Low	Complex	Low	Low	Low	Unproblematic	No	No
Electron microscopy	No	Slow	High	Complex	Low	Medium	Low	Unproblematic	No	No
Enzyme-linked immunosorbent assay (ELISA)	No	Medium	Low	Simple	High	High	High	Unproblematic	No	Yes
Lateral flow test	Yes	Fast	Low	Simple	Low	High	Medium	Unproblematic	No	No
Polymerase chain reaction (PCR)	No	Fast	Low	Simple	Medium	Very high	Medium	Problematic	Limited	No
Quantitative or real-time PCR (qPCR)	No	Fast	Medium	Medium	Medium	Very high	High	Possible	Limited	Yes
Nucleic acid spot hybridization (NASH)	No	Medium	Medium	Simple	Low	Very high	Medium	Unproblematic	Yes	No
Helicase dependent amplification (HDA)	No	Fast	Low	Simple	Medium	Very high	High	Possible	No	No
Recombinase polymerase amplification (RPA)	Yes	Fast	Low	Simple	Medium	Medium	Medium	Possible	No	Yes
Nucleic acid sequence based amplification (NASBA)	No	Fast	Low	Simple	Medium	Very high	High	Possible	No	No
Rolling circle amplification (RCA)	No	Fast	Low	Simple	Medium	Very high	High	Possible	No	No
Loop-mediated isothermal amplification (LAMP)	Yes	Fast	Low	Medium	Medium	Very high	High	Possible	No	Yes
Luminex xMAP	No	Medium	Medium	Medium	High	Very high	High	Unproblematic	Yes	Yes
Luminex xTAG	No	Medium	Medium	Medium	High	Very high	High	Possible	Yes	Yes
Next-generation sequencing (NGS)	No	Slow	High	Complex	High	Very high	High	Unproblematic	Yes	Yes

1.3 Objectives

Plant pathogens can be present in plant propagation material (seeds, seed potatoes, young plants, scions and rootstocks) without showing obvious symptoms. When such infected material is distributed or even imported/exported, plant pathogens can spread and cause severe damages. To prevent this, routine testing for plant pathogens is essential before seeding or planting of the material. Sometimes different pathogens can be present so that several tests have to be performed. In such cases a high-throughput multiplex detection method would be desirable for routine laboratories. When this study was started, such a method did not exist for the diagnosis of plant diseases. The Luminex xMAP and xTAG technologies looked promising and had already been applied in human medicine for such test procedures. Work had already been started to adapt the xMAP technology to plant virus diagnostics. Such an antibody-based technique necessitates the production of specific antibodies and these sometimes do not allow a clear distinction of pathogens. The nucleic acid based xTAG technology can be adapted to clearly differentiate pathogens and does not involve a complicated production of ligands.

The first aim of this study was to adapt the Luminex xTAG technology for plant virus detection. As targets the important CMV and the relevant *Tospovirus* genus were selected. CMV infects many plants including various horticultural and agricultural crops and is counted into the top ten of economically important plant viruses. Tospoviruses are an emerging group of viruses, which spread increasingly around the world and infect more and more plant species. This genus includes TSWV that affects many horticultural and agricultural crops and also belongs to the economically most important plant viruses. Other viruses of this genus are getting more important as they spread to new regions and plants. The developed tests allowed on the one hand a general detection of CMV combined with a distinction of its subgroups I and II and on the other hand a general detection of tospoviruses combined with the differentiation of the important *Tospovirus* species TSWV, INSV, CaCV and WSMoV. The tests were verified with different CMV isolates and *Tospovirus* species and isolates obtained from the DSMZ.

In the second part CMV was further characterized. The CP genes of the tested isolates were cloned and sequenced. Sequences were compared by alignments and phylogenetic analyses. Additionally, infectious full-length clones were created of four CMV isolates belonging to all three subgroups IA, IB and II and differing in symptomatology on *N. benthamiana* plants. These full-length clones produced the same symptoms on *N. benthamiana* as the original isolates and were used for further characterization of CMV, also on the additional host plant *C. sativus*. The nucleotide sequences of the three genomic RNAs of all four isolates were determined. Pseudorecombinants of the isolates were created to narrow down the symptom determinants for the different but strong symptoms of three isolates on *N. benthamiana* and for mosaic symptoms on *C. sativus, respectively*.

2 Development of a molecular assay for the detection of *Cucumber mosaic virus* and the discrimination of its subgroups I and II

Niklas Bald-Blume[1], Jan Bergervoet[2], Edgar Maiss[1]

[1]Department of Phytomedicine, Institute of Horticultural Production Systems, Leibniz Universität Hannover, Hannover, Germany
[2]Plant Science Group, Wageningen University and Research Centre, Wageningen, The Netherlands

2.1 Abstract

A nucleic acid based test for the detection of the economically important plant virus *Cucumber mosaic virus* (CMV) based on the Luminex xTAG technology was developed. This technology has the advantage of allowing the simultaneous detection of various targets. Applying this method, we prove for the presence of CMV in general and differentiate between its two subgroups I and II for which significant differences concerning severity of symptoms and virulence have been reported. For the development of the test procedure the coat protein gene sequences of 29 CMV isolates were cloned and sequenced. Additionally, a subgroup specific ELISA was conducted for comparison. This work is part of a project which aims to develop a test for the simultaneous detection of various plant pathogens (viral, bacterial and fungal) in plant material.

2.2 Introduction

Cucumber mosaic virus (CMV) is a member of the genus *Cucumovirus* in the family *Bromoviridae* (Bujarski et al., 2012). It has the widest host range of known plant viruses affecting more than 1000 species in 85 families worldwide (Roossinck, 2001) including many different crops. This grants it a place in the top ten list of economically important plant viruses (Rybicki, 2015). More than 80 species of aphids are able to transmit this virus in a nonpersistent manner (Gallitelli, 2000). CMV is also spread mechanically, by the parasitic plant dodder (*Cuscuta* spp.) (Schmelzer, 1957) and with varying success through seeds (Yang et al., 1997; Palukaitis et al., 1992). The genome is tripartite and consists of three essential single-stranded plus-sense RNAs (RNA 1, 2 and 3) distributed over three icosahedral particles (Peden and Symons, 1973). Two major subgenomic RNAs (RNA 4 and 4A) are also produced (Schwinghammer and Symons, 1977; Ding et al. 1994). The 5’ ends have a cap and the 3’ ends a tRNA-like structure (Symons, 1975; Ahlquist et al., 1981). Virus particles have a diameter of 30 nm and are made up of RNAs and 180 identical coat protein (CP) subunits with a molecular mass of 24.5 kDa each (Finch et al., 1967; van Regenmortel et al., 1972). CMV can be distinguished into the two subgroups I and II based on serology, nucleic acid hybridization and peptide mapping of the CP (Devergne and Cardin, 1975; Edwards and Gonsalves, 1983; Owen and Palukaitis, 1988; Palukaitis et al., 1992). On the basis of viral nucleic acid sequences subgroup I can be further divided into subgroups IA and IB (Roossinck et al., 1999).

The Enzyme-linked Immunosorbent Assay (ELISA) has developed into the standard method to detect and identify plant viruses (Voller et al., 1976). The virus particles interact with specific antibodies and coupled enzymes produce a color change or another detectable signal. This is a robust and easy to handle

technique but it has the disadvantages that it is quite time and labor intensive and that it only allows to test for one virus at a time.

The Luminex xTAG technology is a nucleic acid based assay which allows the simultaneous detection of up to 500 analytes in one sample. Target DNA is directly amplified by a PCR or if RNA is the target, it is first transcribed into cDNA and subsequently amplified (RT-PCR). The PCR products are then subjected to a target specific primer extension (TSPE) reaction during which biotinylated dCTP is integrated. Later the fluorophore reporter protein streptavidin-R-phycoerythrin can bind to the incorporated biotin. TSPE primers have coupled oligonucleotide tags which serve to hybridize the TSPE products to complementary anti-tags attached to Luminex MagPlex-TAG Microspheres in the next step. These polystyrene microspheres or "beads" are filled with a mix of two to three fluorescent dyes at different ratios creating sets of uniquely color-coded beads with specific oligonucleotide anti-tags. Additionally, the beads are impregnated with magnetite particles allowing their magnetic separation from surrounding buffer and simplifying washing steps. The beads with the hybridized DNA are analyzed in Luminex xMAP systems which use a green laser (532 nm) to excite streptavidin-R-phycoerythrin and a red laser (635 nm) for the bead dyes (van der Vlugt et al., 2015; Rao et al., 2004). In human medicine this technology is already used in diagnostics for example for respiratory viruses (Mahony et al., 2007). In the last years this platform was adopted for the detection of plant pathogens. It has been used to detect and distinguish between different *Pospiviroids* (van Brunschot et al, 2014b) and different begomoviruses and their whitefly vectors (van Brunschot et al., 2014a). A related assay platform utilizing coupled antibodies instead of oligonucleotides, the Luminex xMAP technology, has been applied to identify *Potato virus X* (PVX), *Potato virus Y* (PVY) and *Potato leaf roll virus* (PLRV) (Bergervoet et al., 2008).

In this study a test for CMV in general and for the subgroups I and II based on the Luminex xTAG technology was developed. As test material 29 CMV isolates were obtained from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (DSMZ). The CP genes of these virus isolates were cloned into plasmids for further research and sequenced to get a first impression of their subgrouping. The sequences were stored in the GenBank of the National Center for Biotechnology Information (NCBI). An ELISA with antibodies against CMV subgroups I and II was performed to verify their subgrouping and as comparison for the Luminex xTAG test. Only the discrimination of subgroups I and II was thought to be of interest for this test, because they show differences concerning severity of symptoms and virulence with subgroup I isolates in general being more virulent and causing stronger symptoms (Carrère et al., 1999; Wang et al., 2002). Subgroups IA and IB do not show significant differences in these fields, but they vary in geographic distribution with subgroup IB isolates being mainly found in Asia (Roossinck, 2002; Lin et al., 2003).

2.3 Materials and Methods

2.3.1 Virus specimens

Twenty-nine CMV isolates were obtained from the DSMZ as infected, dried plant material. Information about their origin (host plant, country and provider) was supplied by the DSMZ for most isolates (Table 2.2). This material was used to inoculate healthy *Nicotiana benthamiana* plants by grinding part of it in phosphate buffer (0.05 M KH_2PO_4, 0.05 M Na_2HPO_4, 1 mM EDTA, 5 mM Na-DIECA) with celite and

charcoal and rubbing the material on the leaves of healthy plants. The inoculated leaves were rinsed with water after a few minutes to remove excess buffer and plant debris from the inoculation material.

2.3.2 Nucleic acid extraction

Total RNA was extracted from inoculated and healthy control plants using the RNeasy Plant Mini Kit (Qiagen, Germany) following the kit's instructions.

2.3.3 Primer design

Table 2.1 Characteristics of oligonucleotide primers used in this study. The TSPE primer names are preceded by a 't'. Sequences of cloning primers complementary to vector pGEM-T Easy and tag sequences of TSPE primers are italicized. For the TSPE primers the corresponding microspheres are listed.

Name	Sequence (5'-3')	Length [nts]	Amplicon size [bp]	Corresponding microsphere
CMV_CPs	*CGGCCGCGGGAATTCGAT*-TATGGATGCTTCTCCGCGA	37	810	
CMV_CPas	*CGCGAATTCACTAGTGAT*-TCCAACTCAGCTCCCGCCA	37		
EasyS	ATCACTAGTGAATTCGCGGCCGCCTGCA	28	3020	
EasyAS	ATCGAATTCCCGCGGCCGCCATG	23		
M13U43	AGGGTTTTCCCAGTCACGACGTT	23	1080	
M13R49	GAGCGGATAACAATTTCACACAGG	24		
CMV_RNA1s	CYCTGTAAAAYWACCCTTTG	20	420	
CMV_RNA1as	RTGTGTGACCCAACTTCC	18		
CMV_RNA3s	GTAATCTWACCACYKTSTKT	20	1980	
CMV_RNA3as	TTAGKGACYTCAGRYAGTTT	20		
Nad5s	GATGCTTCTTGGGGCTTCTTGTT	23	180	
Nad5as	CTCCAGTCACCAACATTGGCATAA	24		
tCMV_GENs	*TTCAATTCAAATCAAACACATCAT*-GTAGCCTCCCACGGCGATAAAG	46	270	MTAG-A064
tCMV_GENas	*ACAAATATCTAACTACTATCACAA*-GTCTAAGACGAGGTCCTGCCG	45		MTAG-A039
tCMV_SGIs	*CATAAATCTTCTCATTCTAACAAA*-TCAACCAGTGCTGGTCGTAA	44	630	MTAG-A075
tCMV_SGIas	*CACTACACATTTATCATAACAAAT*-CCACAGAGATCGGAGGGA	42		MTAG-A042
tCMV_SGIIs	*TTAACAACTTATACAAACACAAAC*-GATCTCCCAATGCTAGTAGAAC	46	660	MTAG-A053
tCMV_SGIIas	*ATACTTTACAAACAAATAACACAC*-CGATTGAGAGTGTAGTTTAACG	46		MTAG-A019
tNad5	*AACTTTCTCTCTCTATTCTTATTT*-AGGATCCGCATAGCCCTCGATTTATGTG	52		MTAG-A043

Primers for general detection of CMV by RT-PCR, for cloning of the CMV CP into the vector pGEM Easy, for pre-amplification and for TSPE reactions were designed on the basis of alignments of known CMV nucleotide sequences. Sequences for RNA 1 and RNA 3 were downloaded from the GenBank of the NCBI and imported into the program CLC Main Workbench (CLC bio, Denmark). With the help of this program alignments of the two RNAs were calculated. For the general detection and cloning primers (CMV_CPs/as), the general pre-amplification primers CMV_RNA1s/as and CMV_RNA3s/as and the generic TSPE primers tCMV_GENs/as the alignments were analyzed for conserved regions and corresponding sequences were chosen for the primers. In case of the pre-amplification primers some degenerations were inserted into their sequences. For the subgroup specific TSPE primers tCMV_SGIs/as and tCMV_SGIIs/as conserved regions for each of the subgroups were identified in the alignments and relevant sequences used for the primers. Sequencing primers M13U43 and M13R49

(Stephan, 2005) and primers EasyS and EasyAS for the linearization of the vector pGEM Easy had already been designed from the vector sequence. Pre-amplification primers (Nad5s/as) for the mitochondrial NADH dehydrogenase subunit 5 gene (*nad5*) from Menzel et al. (2002) and TSPE primer (tNad5) for *nad5* from van Brunschot et al. (2014a) adapted from Botermans et al. (2013) were used as plant internal control primers. The tag sequences were attributed to all TSPE primers by Luminex (USA) complementary to anti-tags on the microspheres' surfaces. The characteristics of all primers are listed in Table 2.1 and they were synthesized by Eurofins Scientific (Luxembourg).

2.3.4 Cloning and sequencing of the CP genes

Purified RNA was used in a RT-PCR with primers CMV_CPs/as to amplify the CP gene of all 29 CMV isolates with about 70 nucleotides upstream of the 5'-end and about 30 nucleotides downstream of the 3'-end of the CP gene. RNA was transcribed into cDNA by incubation of 4 µl water, 2 µl purified RNA, 1 µl primer CMV_CPas (10 µM) and 0.5 µl dNTP mix (10 mM each; Thermo Fisher Scientific, USA) at 99 °C for 3 min, followed by rapid cooling on ice and addition of 0.5 µl RevertAid Reverse Transcriptase (20 U/µl; Thermo Fisher Scientific), 2 µl 5X Reaction Buffer (Thermo Fisher Scientific) and incubation at 42 °C for 60 min. The PCR reaction mix consisted of 10 µl Phusion Flash High-Fidelity PCR Master Mix (Thermo Fisher Scientific), 6 µl water, 2 µl cDNA, 1 µl primer CMV_CPs and 1 µl primer CMV_CPas. The PCR program consisted of one cycle for 15 s at 98 °C, 34 cycles of 5 s at 98 °C, 5 s at 60 °C and 15 s at 72 °C and a final extension for 5 min at 72 °C. PCR products were analyzed by gel electrophoresis after adding 4 µl loading buffer (37.5 % glycerol, 0.2 % bromophenol blue, 125X GelRed (Biotium, USA)) and expected fragments of about 800 bp were excised from the agarose gel and eluted using the NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel, Germany) following the manufacturer's instructions. The pGEM Easy vector was linearized and amplified by PCR with the primers EasyS/AS under conditions like above with only the elongation period prolonged for 1 min at 72 °C in all cycles. After gel electrophoresis the corresponding fragment of about 3000 bp was purified. The CP genes were introduced into the vector by Gibson Assembly (Gibson, 2009) resulting in pGEM-T Easy plasmids with inserted CMV CP genes and about 100 additional CMV nucleotides surrounding the CP gene. The CMV-CP primers added an additional thymine and adenine to the pGEM Easy plasmid flanking the insert, resulting in a vector essentially looking like the pGEM-T Easy vector. Vectors with inserts were transformed into *Escherichia coli* strain NM522 following the procedure of Hanahan (1983) and bacteria were cultivated and selected by ampicillin resistance and blue-white screening. Bacterial clones were checked for the correct insert size by restriction digest with the endonucleases *Eco*RI and *Pst*I producing calculated fragments of 18 bp, 826 bp and 2979 bp for vectors with CP inserts. Vectors without insert miss the 826 bp fragment and have an additional 18 bp fragment. Afterwards, positive clones were sequenced with the primers M13U43 or M13R49 flanking the insert on both sides of the vector at Seqlab Sequence Laboratories Goettingen (Germany). Sequences were prepared using the Sequin software tool and submitted to the GenBank of the NCBI.

2.3.5 Pre-amplification RT-PCR

Viral RNA was transcribed into cDNA and then amplified using the Access RT-PCR System kit (Promega, USA) applying the concentrations specified by the manufacturer in 25 µl reactions. The following incubations were carried out: 45 min at 45 °C, 2 min at 94 °C, 30 cycles of 30 s at 94 °C, 30 s at 49 °C and 2 min at 68 °C and a final extension for 7 min at 68 °C. As primers for this pre-amplification rather unspecific degenerate primers were used that enabled the production of an approximately 400 bp

fragment of RNA 1 (CMV_RNA1s/as) and a roughly 2000 bp fragment of RNA 3 (CMV_RNA3s/as) of all CMV isolates. After RT-PCR 5 µl of each product was stained with 1 µl loading buffer (37.5 % glycerol, 0.2 % bromophenol blue, 125X GelRed) and loaded on an agarose gel (1 %) to check for the expected fragments.

2.3.6 TSPE reaction

The pre-amplification products were used for a multiplex TSPE reaction. This reaction is similar to a PCR, but the extension of the primers is the essential step. So the amplification of only one strand would be sufficient and hence the amplification would be linear and not exponential. Instead of normal dCTP Biotin-14-dCTP (Thermo Fisher Scientific) was used together with the remaining unmodified nucleotides (dATP, dGTP, dTTP; Thermo Fisher Scientific). A set of seven primers was used, two generic primers to detect all CMV isolates (tCMV_GENs/as), two specific ones to identify only the subgroup I isolates of CMV (tCMV_SGIa/as), another specific pair for the subgroup II isolates (tCMV_SGIIs/as) and one internal control primer (tNad5) for *nad5*. These TSPE primers have a chimeric design with a virus-specific sequence and an oligonucleotide tag complementary to the anti-tag bound to the microspheres. Of the pre-amplification products 5 µl were used in TSPE mixes of 20 µl (0.75 U Platinum GenoType Tsp DNA Polymerase (Thermo Fisher Scientific), 5 µM Biotin-14-dCTP, 5 µM each of normal dATP, dGTP and dTTP, 1.5 mM $MgCl_2$, 25 nM of each primer, 1X PCR Rxn Buffer (Thermo Fisher Scientific), sterile water up to 20 µl). The reaction mix was incubated under the following conditions: One cycle for 2 min at 94 °C, 35 cycles of 0.5 min at 94 °C, 1 min at 55 °C and 2 min at 72 °C and a final extension for 7 min at 72 °C.

2.3.7 Microsphere hybridization

The TSPE products were hybridized to the corresponding beads. A mix of the seven Luminex MagPlex-TAG Microspheres MTAG-A064, MTAG-A039, MTAG-A075, MTAG-A042, MTAG-A053, MTAG-A019 and MTAG-A043 (0.5 µl of each bead per reaction containing 1250 beads) was prepared in 2X Tm hybridization buffer (0.4 M NaCl, 0.2 M Tris, 0.16 % Triton X-100, pH 8.0; 25 µl per reaction) and double distilled and deionized water (16.5 µl per reaction). Aliquots of the mixture were loaded into 96-well polycarbonate microplates (Corning, USA). Afterwards 5 µl of the TSPE products were added to each well for a final volume of 50 µl with 25 microspheres each per µl hybridization mixture. Each of the microspheres is filled with a distinct fluorescent dye mix and has a specific oligonucleotide anti-tag bound to its surface. This anti-tag is complementary to the tag sequence incorporated into the TSPE primers. The interaction between the complementary tag and anti-tag sequences allows the hybridization of TSPE products to the microspheres. For this the mixtures are first denatured at 96 °C for 90 s and then incubated at 37 °C for 30 min.

2.3.8 Luminex assay

The content of a 96-well polycarbonate microplate was moved to a Cellstar 96-well cell culture plate (Greiner Bio-One, Austria) and the microspheres were pelleted by placing the plate on a magnetic separator for 1 min. The supernatant was discarded and the beads were resuspended and washed two times in 80 µl 1X Tm hybridization buffer. The supernatant was removed again and 80 µl 1X Tm hybridization buffer containing streptavidin-R-phycoerythrin (2 µg/ml; Thermo Fisher Scientific) were added to the beads. The plates were protected from light and incubated on a shaker (600 rpm) at room temperature for 15 min. After this the beads were pelleted again, the supernatant discarded and the microspheres resuspended in 90 µl 1X Tm hybridization buffer. Finally 70 µl of the microsphere

suspensions were analyzed in a Luminex 200 System with the xPONENT Software (Version 3.0; Luminex). In this instrument the beads with the bound TSPE products flow individually through a thin capillary and pass two lasers. A red laser excites the unique dye in each bead and a green laser does the same for the R-phycoerythrin which is bound via streptavidin to the biotin-dCTP of the TSPE products. The resulting fluorescence of the R-phycoerythrin is recorded as the median fluorescence intensity (MFI) signal which allows the positive or negative evaluation for the presence of amplified CMV nucleic acids in the samples. While the fluorescence of the beads allows their identification and grouping.

2.3.9 ELISA

A Triple Antibody Sandwich ELISA (TAS-ELISA) (Clark and Adams, 1977) against CMV subgroup I and II was conducted to verify the grouping with that of the Luminex assay. ELISA test kits against CMV subgroup I (SRA44700) and CMV subgroup II (SRA44800) were obtained by LINARIS Biologische Produkte (Germany) including capture antibodies, detection antibodies and alkaline phosphatase conjugated antibodies. The test was performed following the instructions of the manufacturer with some adaptations. A blocking step with 2 % skim milk in PBS-T was added after the coating of the plates with the coating antibody. Nunc-Immuno 96-well MaxiSorp plates (Thermo Fisher Scientific) were utilized instead of the supplied plates. A sample extraction buffer (0.05 M Tris, 0.06 M Na_2SO_3, pH 8.5) was used instead of the general extraction buffer described in the instructions to extract virus particles from infected plants. Also the antibodies were diluted 1:300 in case of CMV I and 1:200 for CMV II instead of 1:100.

2.4 Results

2.4.1 Cloning of the CP genes, sequencing and phylogenetic analysis

N. benthamiana plants were successfully infected by all 29 CMV isolates through mechanical inoculation. Infection was verified by RNA extraction, RT-PCR and subsequent gel electrophoresis. Symptoms on the plants were caused by 19 of the isolates. The remaining ten isolates were symptomless (Table 2.2). The CP genes of all isolates were amplified, cloned into the vector pGEM-T Easy and sequenced. Alignments of the nucleotide sequences and the deduced amino acid sequences were created and compared (data not shown). Also a phylogenetic tree was constructed from the nucleotide sequences to determine the relationship of the isolates. Reference CP gene sequences of CMV strains Tfn for subgroup IB (accession number Y16924), Fny for subgroup IA (accession number NC_002034) and LS for subgroup II (accession number AF416899) were used for subgrouping. The CP gene sequence of *Peanut stunt virus* isolate PSV ER (accession number U15730) was used as an outgroup (Fig. 2.1). According to the sequencing results and the generated phylogenetic tree, 20 isolates belonged to subgroup I and nine isolates to subgroup II. Almost all subgroup I isolates showed symptoms, while the subgroup II isolates were mainly symptomless, with some exceptions: Two subgroup I isolates caused no symptoms (PV-0189 and PV-0185) and one subgroup II isolate depicted visible symptoms (PV-0418). Of the subgroup I isolates nine could be further classified into subgroup IA and eleven into subgroup IB (Table 2.2 and Fig. 2.1). Nucleotide sequences of the 27 CMV isolates for which no CP sequences existed were stored in the GenBank with new accession numbers (Table 2.2). For two isolates (PV-0418 and PV-0187) sequences are found in GenBank. For isolate PV-0418 the CP gene with surrounding nucleotides is supplied in the GenBank under accession number AJ810256 (Deyong et al., 2005) and a partial 2a and 2b gene under FN555202. For isolate PV-0187 the sequences of all three

RNAs are found under accession numbers KP165580 (RNA 1), KP165581 (RNA 2) and KP165582 (RNA 3) including the CP gene on RNA 3. Our CP gene sequences for the two isolates differed from these existing sequences in three positions for isolate PV-0418 and in four positions for PV-0187.

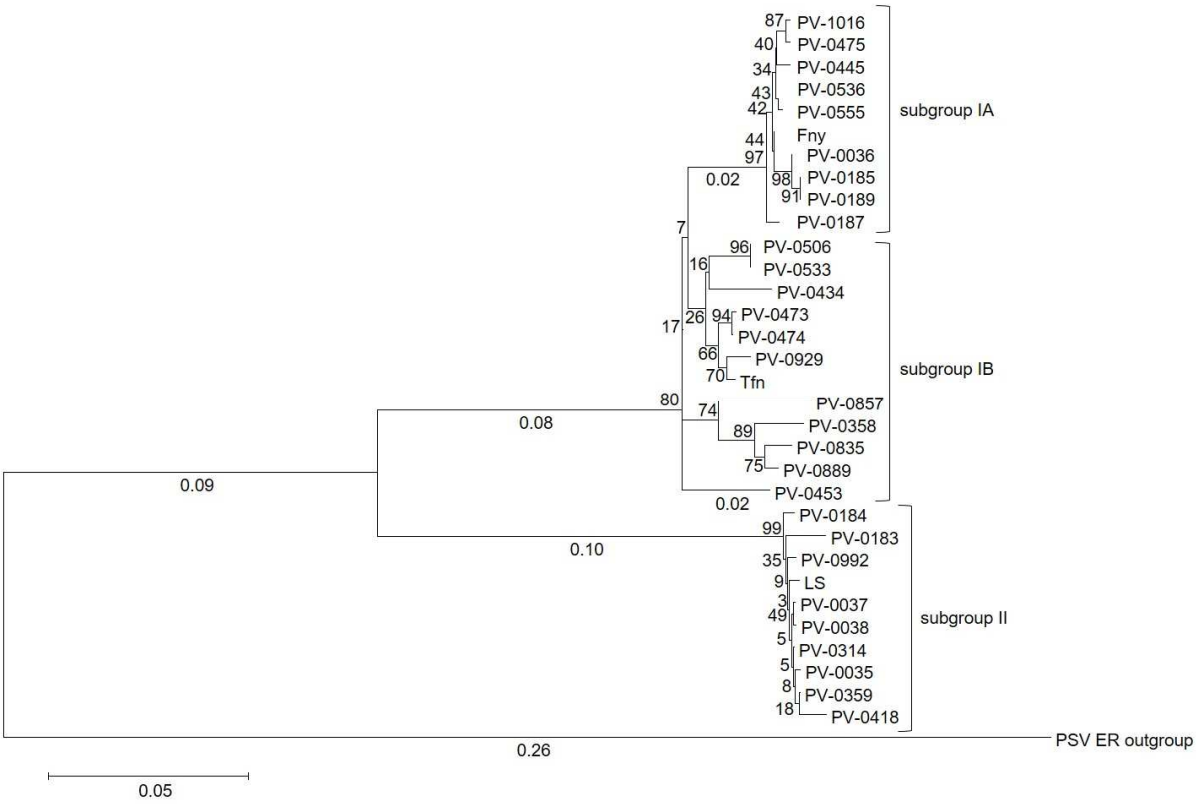

Fig. 2.1 Relationship of the CP gene sequences of the 29 CMV isolates, reference strains and PSV ER as outgroup. The optimal tree with the sum of branch length = 0.74724214 was inferred using the Neighbor-Joining method (Saitou and Nei, 1987) in MEGA6 (Tamura et al., 2013). Bootstrap values (2000 replicates) are shown next to the branches (Felsenstein, 1985). The tree is drawn to scale and the evolutionary distances were computed as number of base substitutions per site using the Maximum Composite Likelihood method (Tamura et al., 2004). There was a total of 657 positions in the final dataset with all positions containing gaps and missing data being eliminated.

Table 2.2 CMV isolates from the DSMZ. Information about the host plant, the country of origin and the provider was given by the DSMZ for most isolates. The other characteristics - symptomatology, subgrouping according to different methods and accession numbers - are results of this study except for the accession numbers of isolates PV-0418 and PV-0187, for which sequences with corresponding accession numbers in italics were already available in GenBank. Isolates PV-0185 and PV-0189 could not be classified into subgroups by ELISA and isolate PV-0835 fell slightly short of the threshold for subgroup I. Also isolate PV-0185 could not be placed into a subgroup by the Luminex test.

CMV isolate	Host plant	Country of origin	Provider	Symptoms on *N. benthamiana*	CP gene sequence subgroup	ELISA subgroup	Luminex subgroup	GenBank accession number
PV-1016	unknown	unknown	H.-J. Vetten	yes	IA	I	I	KU976463
PV-0992	*Buddleya sp.*	Germany	W. Menzel	no	II	II	II	KU976464
PV-0929	*Solanum lycopersicum*	Vietnam	Hueng	yes	IB	I	I	KU976465
PV-0889	*Capsicum annuum*	Iran	unknown	yes	IB	I	I	KU976466
PV-0857	*Capsicum sp.*	Sudan	S. Winter	yes	IB	I	I	KU976467
PV-0835	*Corylus avellana*	Iran	M. Koohi Habibi, Moeini	yes	IB	I?	I	KU976468
PV-0555	*Solanum lycopersicum*	Italy	P. Roggero	yes	IA	I	I	KU976469
PV-0536	*Cucumis melo*	Italy	Tomassoli	yes	IA	I	I	KU976470
PV-0533	*Capsicum sp.*	Nigeria	G. Thottappilly	yes	IB	I	I	KU976471
PV-0506	*Solanum lycopersicum*	China	K.H. Hellwald	yes	IB	I	I	KU976472
PV-0475	unknown	France	H.-J. Vetten, H. Lot	yes	IA	I	I	KU976473
PV-0474	*Cucumis melo*	Spain	L. Arteaga	yes	IB	I	I	KU976474
PV-0473	*Phaseolus vulgaris*	Italy	IFA	yes	IB	I	I	KU976475
PV-0453	*Cucurbita pepo*	Taiwan	S.K. Green	yes	IB	I	I	KU976476
PV-0445	*Passiflora edulis*	Nigeria	G. Thottappilly	yes	IA	I	I	KU976477
PV-0434	*Musa sp.*	Nigeria	S. Winter	yes	IB	I	I	KU976478
PV-0418	unknown	USA	R. Campbell	yes	II	II	II	*AJ810256*
PV-0359	unknown	Iran	H.-L. Weidemann	no	II	II	II	KU976479
PV-0358	unknown	Iran	H.-L. Weidemann	yes	IB	I	I	KU976480
PV-0314	*Pelargonium peltatum*	Germany	D.-E. Lesemann, M. Weiß	no	II	II	II	KU976482
PV-0189	unknown	Israel	H.-J. Vetten	no	IA	-	I	KU976483
PV-0187	*Cucumis sativus*	Germany	unknown	yes	IA	I	I	*KP165582*
PV-0185	*Phaseolus vulgaris*	Zambia	H.-J. Vetten	no	IA	-	-	KU976484
PV-0184	*Capsicum sp.*	Australia	R.I.B. Francki	no	II	II	II	KU976485
PV-0183	*Nicotiana tabacum*	unknown	J.M. Kaper	no	II	II	II	KU976486
PV-0038	*Nicotiana tabacum*	unknown	O. Bode	no	II	II	II	KU976487
PV-0037	*Nicotiana tabacum*	unknown	O. Bode	no	II	II	II	KU976488
PV-0036	unknown	unknown	W.C. Price	yes	IA	I	I	KU976489
PV-0035	*Aristolochia sp.*	unknown	Brückbauer	no	II	II	II	KU976490

2.4.2 TAS-ELISA for CMV subgroup discrimination

The TAS-ELISA against subgroup I and II of CMV confirmed the sequence based classification of almost all isolates into one of the two subgroups. Seventeen isolates were tested positive with antibodies against CMV subgroup I coinciding with the 20 isolates classified into subgroup I based on CP gene sequences (black columns in Fig. 2.2). The threshold for both tests was set at two times the absorbance value at 405 nm of the healthy plant in the CMV subgroup I specific test (dashed line in Fig. 2.2). The value for the subgroup II specific test was slightly lower, but was left out of the figure for the sake of clarity. One isolate expected to belong to subgroup I (PV-0835) showed a reaction with the subgroup I specific antibodies, but fell short of the threshold. Isolates PV-0185 and PV-0189, predicted to belong to subgroup I from their CP gene sequences, did not react with any of the antibodies. These isolates could not be ranked among the two subgroups by a TAS-ELISA although their RNA was present and symptoms were visible on the PV-0835 inoculated plants. Isolates PV-0185 and PV-0189 have an amino acid substitution at position 13 of the CP and isolate PV-0835 at position 135 (see discussion). The remaining nine isolates were positively tested with subgroup II specific antibodies as expected (white columns in Fig. 2.2).

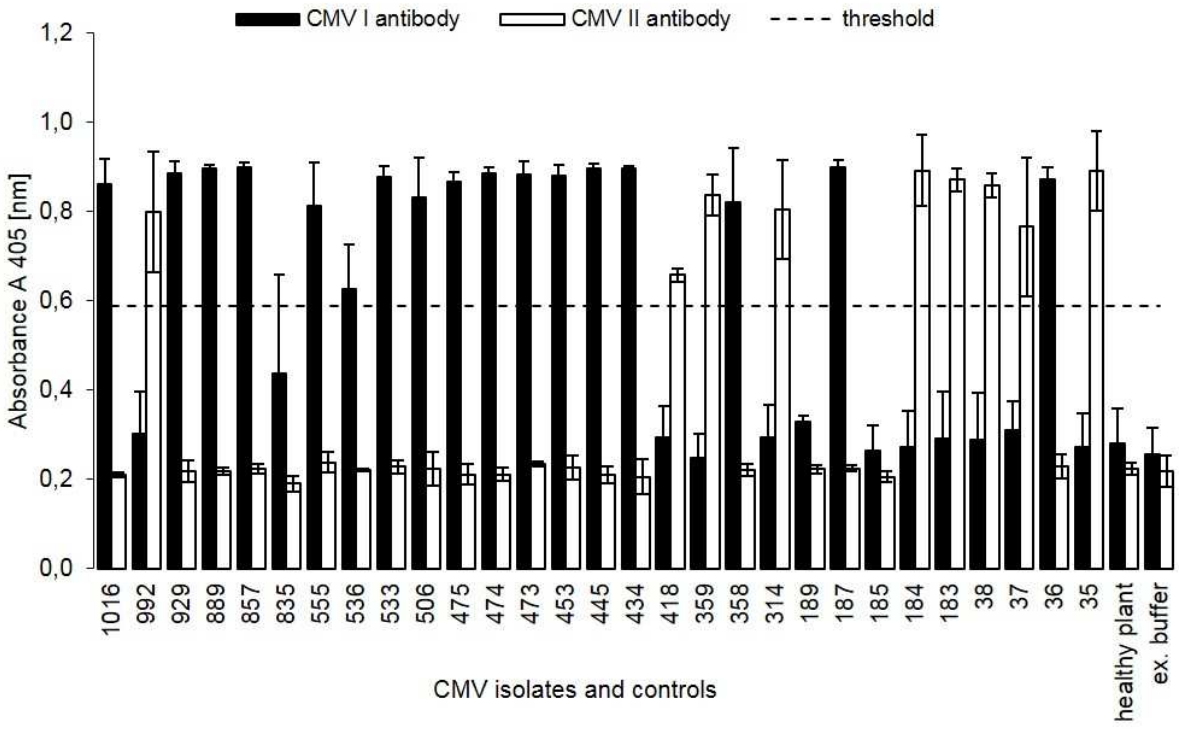

Fig. 2.2 Results of TAS-ELISA tests with specific antibodies against CMV subgroup I or II. Plant material infected with 29 CMV isolates was tested with both antibodies as well as healthy plant material and the extraction buffer as negative controls. Absorbance measurements were taken at a wavelength of 405 nm. The threshold for both tests was set at twice the absorbance value of the healthy plant in the CMV subgroup I test.

2.4.3 RT-PCR analysis of pre-amplification and TSPE primers

The pre-amplification primers CMV_RNA1s/as and CMV_RNA3s/as were successfully used in RT-PCRs producing the expected fragments of about 400 bp for RNA 1 and approximately 2000 bp for RNA 3 for all isolates. The TSPE primers tCMV_GENs/as, tCMV_SGIs/as and tCMV_SGIIs/as were also employed in RT-PCRs with the RNAs of all isolates to test their effectiveness and specificity. The

generic primers tCMV_GENs/as generated expected fragments of about 270 bp for all isolates, while the subgroup specific primers tCMV_SGIs/as induced fragments of roughly 630 bp in the twenty isolates grouped into subgroup I based on CP gene sequences and CMV_SGIIs/as produced fragments of approximately 670 bp in the remaining nine isolates classified in subgroup II resting upon CP gene sequences.

2.4.4 Luminex assay for CMV detection and subgroup discrimination

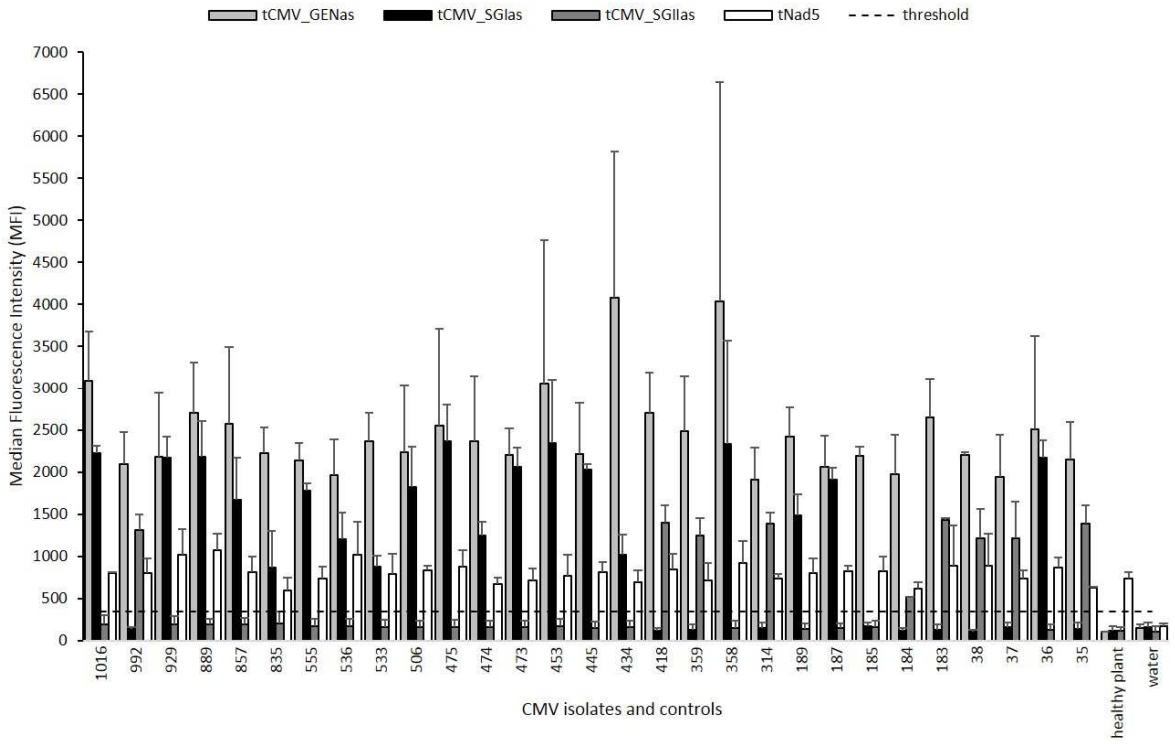

Fig. 2.3 Results of the Luminex assay. The tests were conducted against CMV in general, against subgroup I and II of CMV and against a plant internal control. Plant material infected with 29 CMV isolates was used as well as healthy plant material and water as negative controls. The median fluorescence intensity (MFI) was measured. The threshold for all tests was set at two times the MFI value of the water control of the tNad5 test (dashed line).

All TSPE primers were used together with the pre-amplification products in TSPE reactions. Their products were hybridized to the seven MagPlex-TAG Microspheres MTAG-A064, MTAG-A039, MTAG-A075, MTAG-A042, MTAG-A053, MTAG-A019 and MTAG-A043 and the hybridization mix was then analyzed in three independent Luminex tests. The tCMV_GENas TSPE primer allowed the detection of all 29 CMV isolates together with bead MTAG-A039. The threshold was exceeded in all cases (light grey columns in Fig. 2.3). The subgroup specific TSPE primers tCMV_SGIas and tCMV_SGIIas together with the microspheres MTAG-A042 and MTAG-A019 successfully identified and distinguished between most subgroup I and II isolates. Nineteen out of the 20 isolates classified into subgroup I based on CP gene sequences were detected. Only for isolate PV-0185 the threshold was not exceeded (black columns in Fig. 2.3). In the subgroup II test all nine isolates classified into subgroup II due to CP gene sequences could be identified (dark grey columns in Fig. 2.3). All plant samples including the CMV infected plants and the healthy plant were recognized in the plant internal control test by the tNad5 TSPE primer together with bead MTAG-A043 (white columns in Fig. 2.3). As a threshold for all tests two times the MFI value of the water control of the tNad5 test was selected,

because this sample showed the highest MFI value of all the healthy plant and water controls. The values of the other tests were comparable, but were left out of the figure for the sake of clarity (dashed line in Fig. 2.3). The other three TSPE primers (tCMV_GENs, tCMV_SGIs and tCMV_SGIIs) did not generate satisfactory results in Luminex tests for CMV detection and subgroup discrimination (data not shown). But this was not necessary, as one primer is sufficient for the TSPE reaction and the following Luminex test.

2.5 Discussion

All 29 CMV isolates infected *N. benthamiana* plants causing either no symptoms at all or visible symptoms of different severity. Most of the subgroup I isolates caused symptoms except for isolates PV-0189 and PV-0185 while most of the subgroup II isolates provoked symptomless infections except for isolate PV-0418. These findings are supported by Carrère et al. (1999) who stated that subgroup I strains usually cause severe symptoms, while subgroup II strains show reduced virulence and induce milder symptoms.

The CP genes of all the isolates were cloned and sequenced. From these sequences the subgrouping of the isolates and their phylogenetic relationship was derived. Of course this classification only takes the CP gene on RNA 3 into consideration. CMV RNAs can have independent evolutionary histories with reassortments of their RNAs occurring between CMV subgroups and also between *Cucumovirus* species (Nouri et al., 2014; Roossinck, 2002; White et al., 1995). Additionally, recombinations of parts of a RNA are also possible between CMV subgroups (Bonnet et al., 2005). This means that RNA 1, 2 and parts of RNA 3 could belong to a different subgroup than the sequenced CP gene. Here the classification according to the CP gene is sufficient, because for the TSPE reaction primers were used in the region of the CP gene and the antibodies for the ELISA also interact with the capsid formed by CPs. The obtained sequences of 27 isolates were entered into the GenBank and accession numbers assigned. For two of the investigated isolates (PV-0418 and PV-0187) CP gene sequences already exist in GenBank. Our sequences differed from these in the three nucleotide positions 19, 223 and 415 (A instead of C, C instead of A and G instead of A) of the CP gene of PV-0418 and in the four positions 89, 253, 429 and 654 (T instead of C or C instead of T) of PV-0187 leading to three (PV-0418) or two amino acid substitutions in the CP (PV-0187), respectively. These single nucleotide polymorphisms could be inherent to the virus populations of these isolates or they could be a result of RT-PCR or sequencing errors. Following the quasispecies concept for viruses, mutations always occur so that single viruses can have varying sequences with single nucleotide polymorphisms prevailing (Eigen, 1993).

From the obtained CP gene sequences and already existing RNA 1 and 3 sequences from GenBank a molecular assay using the Luminex xTAG technology was successfully developed. This assay reliably detected all 29 CMV isolates. It successfully discriminated between the virus' subgroups I and II placing almost all isolates into one of the subgroups as predicted by their CP gene sequences. Only one isolate could not be classified into a subgroup using this technique. Isolate PV-0185 did not react with any of the subgroup specific primers in the Luminex test. This happened although the subgroup I specific TSPE primers (tCMV_SGIs/as) showed almost complete congruence of the virus-specific part to the sequence of the CP gene region of this isolate. In addition, RT-PCR experiments with these primers also produced the expected amplicon for this isolate. Our test can be compared with the CMV microarray of Deyong et al. (2005). They developed a hybridization microarray on a glass slide for CMV detection allowing

also the distinction of subgroups I and II and furthermore of the subgroups IA and IB. Fourteen CMV isolates were detected and grouped into either subgroup I or II and these results were confirmed by a subgroup specific ELISA. Most subgroup I isolates could be further divided into subgroups IA and IB except for one isolate. For our study the classification into subgroup IA and IB was not of interest, because these two subgroups only differ in geographic distribution and not in virulence and symptomatology (Roossinck, 2002; Lin et al., 2003).

The results of our Luminex test were supported by the subgroup specific ELISA. Isolates were grouped into the same subgroup I or II, respectively, in the ELISA as well as in the Luminex test. Also isolate PV-0185 could not be classified into any of the subgroups by an ELISA as it did not react with any of the antibodies. The same applies to isolate PV-0189 which was allocated to subgroup I in the Luminex test. The CP gene sequences of these two isolates were compared with the other isolates' sequences and one distinct deviation was identified. At nucleotide positions 34 to 36 the CP gene of the two isolates contains the codon AGC instead of AAC leading to an amino acid substitution of serine instead of asparagine at amino acid position 13 of the coat protein. Isolate PV-0835 reacted with the subgroup I specific antibodies but fell short of the threshold. Here sequence alignments reveal a unique amino acid substitution of methionine instead of leucine at position 135 of the CP resulting from the codon ATG instead of CTG at positions 400 to 402 of the CP gene. These amino acid changes might affect one of the ten epitopes Hsu et al. (2000) identified on CMV virions using monoclonal antibodies. Some of these epitopes are generic and will be found on most CMV isolates, while others are subgroup or even strain specific. Single isolates might have further mutated epitopes prohibiting detection by certain antibodies. Hsu et al. (2000) found that seven out of 109 tested CMV isolates (6 %) did not react with subgroup specific antibodies in ELISA experiments, which is similar to our value of 7 % (two out of 29 isolates). So it is not unusual that some CMV isolates do not interact with subgroup specific antibodies. Furthermore, the point mutations leading to amino acid substitutions in the CP might indeed explain why isolates PV-0185 and PV-0189 were not detected in the ELISA experiments and why isolate PV-0835 only reacted slightly with subgroup I specific antibodies. The amino acid substitution in the CP of isolates PV-0185 and PV-0189 could also be the reason for the failure to cause symptoms.

The developed Luminex xTAG test for CMV can be combined with already existing plant pathogen tests using this platform (van Brunschot et al, 2014a; van Brunschot et al., 2014b) and others that will be devised in the future to create assays for certain crops detecting their most important pathogens similar to the respiratory virus panel test of Mahony et al. (2007) which screens for 20 different human respiratory viruses and their subtypes. Lim et al. (2016) designed such a test revealing the infection and co-infection of lily plants with the three viruses CMV, *Lily mottle virus* (LMoV) and *Lily symptomless virus* (LSV). In theory the Luminex xTAG technology would allow the simultaneous detection and distinction of 500 targets with the supplied 500 distinct microspheres and their specific anti-tags. But in practice this will not be realizable, because of multiplexing limits of RT-PCRs. However, a test for the 20 most important diseases of a crop similar to the one described by Mahony et al. (2007) for human pathogens would already be a substantial progress compared to current methods.

3 Characterization of four infectious *Cucumber mosaic virus* full-length clones expressing symptoms of varying intensity on *Nicotiana benthamiana* plants and investigation of their pseudorecombinants

Niklas Bald-Blume, Sarah Trebing, Edgar Maiss

Department of Phytomedicine, Institute of Horticultural Production Systems, Leibniz Universität Hannover, Hannover, Germany

3.1 Abstract

Different isolates of *Cucumber mosaic virus* express symptoms of varying intensity and appearance on *Nicotiana benthamiana* plants. Full-length clones of four isolates belonging to the three subgroups IA, IB and II and differing in symptomatology were created. These full-length clones were infectious and caused the same symptoms as the original isolates. The nucleotide sequences of the three RNAs of the four clones were determined, phylogenetically analyzed and published in the GenBank of the NCBI. Three clones infected *Cucumis sativus* plants and caused mosaic symptoms on the cultivars Vorgebirgstrauben and Delikatess. Pseudorecombinants of the four isolates were investigated to localize the determinants for their varying symptomatology. For isolate PV-0474 the determinant for shoestring-like leaf deformations on *N. benthamiana* was located on RNA1. All three RNAs of isolate PV-0036 alone with the remaining two RNAs of the mild isolate PV-0184 were able to trigger severe chlorosis on *N. benthamiana*. While for the stunted growth phenotype of *N. benthamiana* induced by isolate PV-0506 a combination with more than one of its RNAs seems to be essential.

3.2 Introduction

Cucumber mosaic virus (CMV) belongs to the genus *Cucumovirus* in the family *Bromoviridae* (Bujarski et al., 2012). With more than 1,000 species in 85 families affected, it has one of the widest host ranges of plant viruses and is distributed worldwide (Roossinck, 2001). Many different crop plants are hosts of this virus, so that it is among the ten economically most important plant viruses (Rybicki, 2015; Scholthof et al., 2011). It is spread by more than 80 species of aphids in a nonpersistent manner (Gallitelli, 2000), by the plant parasitic dodder (*Cuscuta* spp.) (Schmelzer, 1957), with varying success through seeds (Palukaitis et al., 1992; Yang et al., 1997) and mechanically. The genome is composed of three linear plus-sense single-stranded genomic RNAs, denominated RNA1, RNA2 and RNA3, which are indispensable for infection. RNA1 consist of about 3,400 nucleotides, RNA2 of approximately 3,000 nucleotides and RNA3 of roughly 2,200 nucleotides (Gould and Symons, 1982; Rezaian et al., 1984; Rezaian et al., 1985). The 5’ ends of the RNAs have a cap and the 3’ ends a tRNA-like structure (Ahlquist et al., 1981; Symons, 1975). Also two major subgenomic RNAs (RNA4 and RNA4A) are synthesized (Ding et al., 1994; Schwinghamer and Symons, 1977). The 110 kDa 1a protein with a putative methyltransferase and RNA helicase activity is translated from the monocistronic RNA1 (Habili and Symons, 1989; Rozanov et al., 1992). RNA2 encodes the 98 kDa 2a protein, which is the RNA-dependent RNA polymerase (RdRp), and the 15 kDa 2b protein on the subgenomic RNA4A, which is a suppressor of gene silencing and plays a role in virus transport (Guo and Ding, 2002; Hayes and Buck, 1990). RNA3 is also bicistronic encoding the 30 kDa 3a protein, which is the movement

protein (MP), and the 25 kDa 3b protein, which is the coat protein (CP) translated from the subgenomic RNA4 (Ding et al., 1995; van Regenmortel et al., 1972). Both the MP and the CP are necessary for virus movement (Suzuki et al., 1991). For aphid transmission determinants are located in the CP (Chen and Francki, 1990). The genomic and subgenomic RNAs are distributed over three icosahedral particles (Peden and Symons, 1973). These particles have a diameter of 30 nm and consist of RNAs and 180 copies of the CP (Finch et al., 1967). Based on serology, nucleic acid hybridization and peptide mapping of the CP CMV can be differentiated into the two subgroups I and II (Devergne and Cardin, 1975; Edwards and Gonsalves, 1983; Owen and Palukaitis, 1988; Palukaitis et al., 1992). Further subdivision of subgroup I into subgroups IA and IB can be achieved on the basis of nucleic acid sequences (Roossinck et al., 1999). Subgroups I and II vary in severity of symptoms and in virulence. Subgroup I isolates in general cause stronger symptoms and are more virulent (Carrère et al., 1999; Wang et al., 2002). Subgroups IA and IB do not differ in virulence and severity of symptoms, but they have different geographic distributions with subgroup IB isolates being mainly found in Asia (Lin et al., 2003; Roossinck, 2002). Reassortment of the RNAs occurs between the different subgroups and also between *Cucumovirus* species (Chen et al., 2007; Nouri et al., 2014; Roossinck, 2002; White et al., 1995). Recombination of parts of different RNAs is also possible between the subgroups (Bonnet et al., 2005; Carrère et al., 1999; Nouri et al., 2014). Most infected host species develop systemic mosaic symptoms, leaf deformation, stunted growth, chlorosis or necrosis. The severity of symptoms depends on the host species, its genotype (e.g. cultivar), the CMV subgroup and also isolate as well as on environmental conditions varying from no symptoms at all to complete systemic necrosis even leading to plant death (Jacquemond, 2012; Palukaitis et al., 1992). Some CMV strains can contain satellite RNAs that either aggravate or ameliorate the symptoms caused by their helper CMV strain (Kaper and Tousignant, 1984; Piazzolla et al., 1998; Sleat et al., 1994).

In this study infectious full-length clones of four CMV isolates belonging to all three subgroups (IA, IB and II) and expressing symptoms of different severity and appearance were created. These isolates were selected from 29 CMV isolates obtained from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (DSMZ, Germany). The full-length clones were characterized on different host plants concerning their infectivity and symptomatology. Additionally, the nucleotide sequences of all three genomic RNAs of all four clones were determined. Artificial reassortants, called pseudorecombinants, were created by mixing the cloned RNAs of the different infectious full-length clones to investigate the symptom determinants of the three symptomatic isolates on *Nicotiana benthamiana* and *Cucumis sativus* plants.

3.3 Materials and Methods

3.3.1 Virus specimens

The four cloned CMV isolates were part of 29 CMV isolates obtained from the DSMZ as infected, dried plant material. This material was used to mechanically inoculate healthy *N. benthamiana* plants using phosphate buffer (0.05 M KH_2PO_4, 0.05 M Na_2HPO_4, 1 mM EDTA, 5 mM Na-DIECA) with celite and charcoal. The inoculated plants were kept in a greenhouse and monitored for symptom expression. The three isolates PV-0506, PV-0474 and PV-0036 developed strong but different symptoms while isolate PV-0184 was symptomless. These four isolates were used for further experiments. About two weeks after inoculation total RNA was extracted from inoculated plants and screened for RNA of CMV by RT-

PCR. Information about the origin of the four cloned isolates (host plant, country of origin and provider) was supplied by the DSMZ (Table 3.1).

Table 3.1 CMV isolates from the DSMZ. Information about the host plant, the country of origin and the provider was given by the DSMZ for most isolates. The symptomatology, the subgrouping according to genomic sequences and the accession numbers are results of this study.

CMV isolate	Host plant	Country of origin	Provider	Symptoms on *N. benthamiana*	Subgroup	Accession numbers
PV-0506	*Solanum lycopersicum*	China	K.H. Hellwald	stunted growth	IB	KX525728 (RNA1) KX525732 (RNA2) KX525736 (RNA3)
PV-0474	*Cucumis melo*	Spain	L. Arteaga	shoestring leaves	IB	KX525729 (RNA1) KX525733 (RNA2) KX525737 (RNA3)
PV-0184	*Capsicum sp.*	Australia	R.I.B. Francki	No	II	KX525730 (RNA1) KX525734 (RNA2) KX525738 (RNA3)
PV-0036	unknown	unknown	W.C. Price	severe chlorosis	IA	KX525731 (RNA1) KX525735 (RNA2) KX525739 (RNA3)

3.3.2 Nucleic acid extraction

Total RNA was extracted from healthy control plants and from plants inoculated either with the four isolates or their full-length clones as well as their pseudorecombinants using the RNeasy Plant Mini Kit (Qiagen, Germany) following the kit's instructions.

3.3.3 Primer design

Table 3.2 Characteristics of the primers used in this study. Sequences complementary to pDIVA are italicized.

Name	Sequence (5'-3')	Length [nts]
CMV_CPs	TATGGATGCTTCTCCGCGA	19
CMV_CPas	TCCAACTCAGCTCCCGCCA	19
CMV1s	*AGGAAGTTCATTTCATTTGGAGAGG*-GTTTATTTACAAGAGCGTACGGTTCAATCCC	56
CMV2s	*AGGAAGTTCATTTCATTTGGAGAGG*-GTTTATTTACAAGAGCGTACGGTTCAAC	53
CMV3s	*AGGAAGTTCATTTCATTTGGAGAGG*-GTAATCTTACCACTGTGTGTGTGCG	50
CMV123as	*GAGATGCCATGCCGACCC*-TGGTCTCCTTKDRGAGRCCC	38
CMV1Ms	GAATGGGACGTGATATCATC	20
CMV1Mas	GATGATATCACGTCCCATTC	20
CMV1M+Vs	*AGGAAGTTCATTTCATTTGGAGAGG*-GAATGGGACGTGATATCATCAGC	48
CMV1M+Vas	*GAGATGCCATGCCGACCC*-GCTGATGATATCACGTCCCATTC	41
Papa	CCTCTCCAAATGAAATGAACTTCCTTATATAG	32
Mama	GGGTCGGCATGGCATCTCCACCTCCTC	27
Seq_35S	CCAACCACGTCTTCAAAGCAAGTG	24
J2	CCCTTATCTGGGAACTACTCACAC	24
CMV1Seqs	CATGTCTTATGTTCATGATTGGGAG	25
CMV1Seqas	CAGTCGGACATTCATTAAGTAGGAC	25
CMV1seq2as	CATCTTGTGGGCTACGGAATGTG	23
CMV2Seqs	GCTACTGATCGTGTTGATATCAATTTAG	28
CMV2Seqas	TCAACAAAGCTCATGAAGTGAGC	23
CMV2seq2as	GATCAATTCGCACTGACGACGATC	24
CMV3Seqs	GAGGAATTAAATGTTGAGAGCCCTC	25
CMVII1Seqs	GAGGAATCCACGATGTCTTATG	22
CMVII1Seqas	CACAACTGACCAAAGTGCAAC	21
CMVII2Seqs	ATGTAGTTCAAGCCGTTTG	19
CMVII3Seqas	TTAAGAGTGGGACGACCA	18

Primers were designed according to alignments of available sequences of the three RNAs of CMV using the software CLC Main Workbench (CLC bio, Denmark). The characteristics of the primers are listed in Table 3.2. Primers CMV_CPs/as lead to the amplification of the CMV CP gene with surrounding nucleotides and were used to screen for the presence of CMV in RT-PCR tests. The primers contain an additional thymidine at their 5'-ends. The primers CMV1s down to Mama were used for the cloning of the three RNAs of the four isolates into a binary vector (see below). Therefore, some of the primers consist of a virus-specific part and a vector-specific part given in italics in Table 3.2. Primer CMV123as is partly degenerated. The vector-specific primers Seq_35S and J2 were used for amplification of viral inserts for later sequencing. The remaining primers were used for sequencing.

3.3.4 Cloning and sequencing of the RNAs

Purified RNA from healthy control plants and from inoculated plants was used for cDNA synthesis with primer CMV123as allowing the transcription of all three CMV RNAs into cDNA by incubating 4 µl water, 2 µl purified RNA, 1 µl primer CMV123as (10 µM) and 0.5 µl dNTP mix (10 mM each; Thermo Fisher Scientific, USA) at 99 °C for 3 min, followed by rapid cooling on ice and addition of 0.5 µl RevertAid Reverse Transcriptase (20 U/µl; Thermo Fisher Scientific), 2 µl 5X Reaction Buffer (Thermo Fisher Scientific) and incubation at 42 °C for 60 min. Subsequently, the cDNA was used in PCRs with primers CMV123as and either primer CMV1s, CMV2s or CMV3s (each at a concentration of 10 µM) for amplification of cDNA from RNA1, RNA2 or RNA3, respectively. The PCR reaction mix consisted of 10 µl Phusion Flash High-Fidelity PCR Master Mix (Thermo Fisher Scientific), 6 µl water, 2 µl cDNA, 1 µl primer CMV123as and 1 µl of either primer CMV1s, CMV2s or CMV3s. The PCR program was composed of one cycle for 15 s at 98 °C, 34 cycles of 5 s at 98 °C, 5 s at 50 °C and 60 s at 72 °C and a final extension for 5 min at 72 °C. PCR products were analyzed by gel electrophoresis after adding 4 µl loading buffer (37.5 % glycerol, 0.2 % bromophenol blue, 125X GelRed (Biotium, USA)) and expected fragments of about 3.4 kb for RNA1, 3.0 kb for RNA2 or 2.2 kb for RNA3 were excised from the agarose gels and eluted using the NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel, Germany) following the manufacturer's instructions. The PCR fragments of RNA1 to RNA3 of all four isolates were introduced into a modified mini binary vector (Xiang et al., 1999) called pDIVA (accession number KX665539; see Fig. 8.1 in Addendum for vector map) by Gibson Assembly (Gibson et al., 2009) through the overlapping vector sequences added to the primers CMV1s, CMV2s, CMV3s and CMV123as. For this purpose, the mini binary vector was amplified by PCR with the primers Papa and Mama under conditions like above with the annealing temperature increased to 60 °C and the elongation period prolonged to 75 s in all cycles. After gel electrophoresis the corresponding fragment of about 4.3 kb was purified. The purified vector fragment and one of the PCR fragments were assembled and transformed into *Escherichia coli* strain NM522 following the procedure of Hanahan (1983). Transformants were identified by resistance to kanamycin. Plasmids were subsequently isolated from bacteria and checked for inserted PCR fragments by PCR with primers Seq_35S and J2 binding at the CaMV 35S promoter region and at the CaMV pA-signal sequence, respectively. The PCR reaction mix consisted of 5 µl FastGene Taq ReadyMix (NIPPON Genetics EUROPE, Germany), 3 µl water, 1 µl DNA, 0.5 µl primer Seq_35S and 0.5 µl primer J2. The PCR program was composed of one cycle of 3 min at 95 °C, 30 cycles of 30 s at 95 °C, 30 s at 53 °C and 4 min at 72 °C and a final extension for 5 min at 72 °C. Reaction products of 3.7 kb for RNA1, 3.3 kb for RNA2 and 2.5 kb for RNA3 were confirmed by gel electrophoresis after adding 2 µl loading buffer. Plasmids with PCR fragments of the expected size were amplified with Phusion Flash High-Fidelity PCR Master Mix as described above with an

annealing temperature of 60 °C, an elongation period of 60 s and primers Seq_35S and J2. An aliquot of 5 µl of the PCR products was analyzed by gel electrophoresis after adding 1 µl loading buffer. The residual PCR products were purified with SureClean Plus (Bioline, UK) and sequenced with the primers used in the PCR at Seqlab Sequence Laboratories Göttingen (Germany). This cloning strategy worked for all RNAs of all isolates except for RNA1 of isolate PV-0184. This single RNA had to be cloned in two steps. As a first step the 5'-end half and the 3'-end half of the RNA were transcribed, amplified and introduced into pDIVA vector individually. For cDNA synthesis under conditions like above the primer CMV123as for the 3'-end half and the primer CMV1M+Vas for the 5'-end half were used. The PCR was performed as described above with the annealing temperature set at 62 °C and the elongation period decreased to 40 s. As primers the combinations of CMV1s and CMV1M+Vas for the 5'-end half and CMV1M+Vs and CMV123as for the 3'-end half were used. After gel electrophoresis the two corresponding fragments each of about 1.7 kb were eluted and introduced into the vector, which was then transformed into bacteria. As a second step positively selected vectors (PCR as above) containing either the 5'- or the 3'-end half of the RNA from the first step were assembled by a new round of PCR, Gibson Assembly and transformation to obtain a vector with the full RNA1 of PV-0184. Two PCRs were performed as above with the annealing temperature at 60 °C and the elongation period set at 90 s. In one PCR the vector containing the 5'-end half of the RNA was inserted together with primers Mama and CMV1Mas leading to the amplification of the complete vector with the inserted part of the RNA. In the other PCR the vector containing the 3'-end half of the RNA was applied together with primers CMV1Ms and CMV123as leading to the amplification of only the integrated part of the RNA. Because the primers had overlapping sequences, the PCR products could be assembled by Gibson Assembly to yield the complete RNA1 in the pDIVA vector. This vector with the RNA1 of PV-0184 was transformed into bacteria, propagated, isolated, analyzed by PCR and sequenced as described above. Because sequencing with the primers Seq_35S and J2 did not cover the complete cloned RNAs, further sequencing had to be performed with the additional primers CMV1Seqs, CMV1Seqas and CMV1seq2as for RNA1, CMV2Seqs, CMV2Seqas and CMV2seq2as for RNA2 and CMV3Seqs for RNA3 of isolates PV-0506, PV-0474 and PV-0036 as well as the primers CMVII1Seqs and CMVII1Seqas for RNA1, CMVII2Seqs for RNA2 and CMVII3Seqas for RNA3 of isolate PV-0184. Finally, contigs of all RNAs were assembled from the individual sequence files.

3.3.5 Electroporation of *Rhizobium radiobacter* and infiltration of *N. benthamiana* plants

Mixtures of plasmids containing the three cloned RNAs of each isolate were prepared and transferred into *Rhizobium radiobacter* strain GV2260 by electroporation (Mattanovich et al., 1989). An electric pulse of 1.44 kV was applied, cells were moved into 1 ml SOC medium and incubated at 28 °C for three hours. Aliquots of 100 µl were spread on LB plates containing 50 µg/ml kanamycin sulfate and incubated at 28 °C for about 48 hours. Because all three genomic RNAs are necessary for infection with CMV, the infiltration protocol by Zilian and Maiss (2011) and Voinnet et al. (2003) had to be adapted. A mix of the bacteria with all three plasmids of each isolate was created by directly rinsing the bacteria from the LB plates with 12 ml inoculation buffer (10 mM $MgCl_2$, 10 mM MES, 100 µM acetosyringone). The bacterial suspension was then infiltrated into the lower surface of the upper leaves of 4 to 5 weeks old *N. benthamiana* plants using a 2 ml syringe without needle. In the same way pseudorecombinants were created by mixing the plasmids with RNAs of different isolates before electroporation and infiltration. Plants were kept in the greenhouse and monitored for symptom expression. To confirm the presence of CMV, RNA was extracted from plants and RT-PCRs with a

standard cDNA synthesis and FastGene Taq ReadyMix were performed using the primers CMV_CPs/as and the procedures described in 3.3.4 with an annealing temperature of 53 °C and an elongation period of 1 min followed by gel electrophoresis. This was essential to verify the systemic infectivity of the symptomless original isolate PV-0184 and its full-length clone as well as of the symptomless pseudorecombinants.

3.3.6 Propagation of isolates and clones and their infectivity on *C. sativus*

The four CMV isolates and viruses derived from infectious full-length clones were propagated on *N. benthamiana* plants by mechanical inoculation and also on the different *C. sativus* cultivars Vorgebirgstrauben, Delikatess, Bimbostar F1, Darina and Picolino (seeds of Bimbostar F1 from TOM-GARTEN, Germany, seeds of the other cultivars from Bruno Nebelung, Germany). Two to four cucumber plants (primary leaf stage) were inoculated per clone. Plants were kept in the greenhouse and symptom development was observed. Inoculated plants were checked for CMV by RNA extraction and RT-PCR as described in 3.3.5.

3.3.7 Pseudorecombinants of the four isolates

Pseudorecombinants of the four CMV isolates were generated by mixing the plasmids containing the cloned RNAs of different isolates. Because the isolate PV-0184 was symptomless on *N. benthamiana* and did not infect C. *sativus* plants, it was used as the basis of the experiments. This isolate contributed two cloned RNAs to the pseudorecombinants, which was supplemented by a third cloned RNA from one of the three symptom inducing isolates. The pseudorecombinants are denoted with a three number code after the isolate background of their RNA1 (1st number), RNA2 (2nd number) and RNA3 (3rd number). A “1” stands for isolate PV-0184, a “3” for PV-0036, a “4” for PV-0474 and a “5” for PV-0506. For example, the pseudorecombinant denominated 511 consisted of RNA1 from PV-0506 and both RNA2 as well as RNA3 from PV-0184 (Table 3.3). As an exception pseudorecombinant 144 was created containing only RNA1 of PV-0184 and both RNA2 as well as RNA3 of PV-0474.

3.3.8 Semi-quantitative enzyme-linked immunosorbent assay (ELISA)

The approximate virus titers of the full-length clones and the pseudorecombinants 411 and 144 in *N. benthamiana* plants were determined by a semi-quantitative ELISA. The assay format was a Double Antibody Sandwich (DAS)-ELISA (Clark and Adams, 1977) with antibodies against CMV DTL/ToRS (07108S) corresponding to both subgroups I and II of CMV from LOEWE Biochemica (Germany) following the instructions of the manufacturer with some minor adaptations. The test volume was reduced to 100 µl per well. Samples were prepared by grinding 100 mg plant material in 600 µl Sample Buffer (PBS-TPO), centrifuging for 2 min at 13,000 rpm and transferring 150 µl of supernatant into new reaction tubes containing 750 µl sample buffer. Nunc-Immuno 96-well MaxiSorp plates (Thermo Fisher Scientific) were utilized. An alkaline phosphatase coupled to the second antibody converts 4-nitrophenylphosphate into the yellow colored 4-nitrophenol. This color development was measured in a spectrophotometer at 405 nm after two hours with two repetitions.

3.4 Results

3.4.1 Infectivity and symptomatology of the CMV isolates and CMV derived from full-length clones

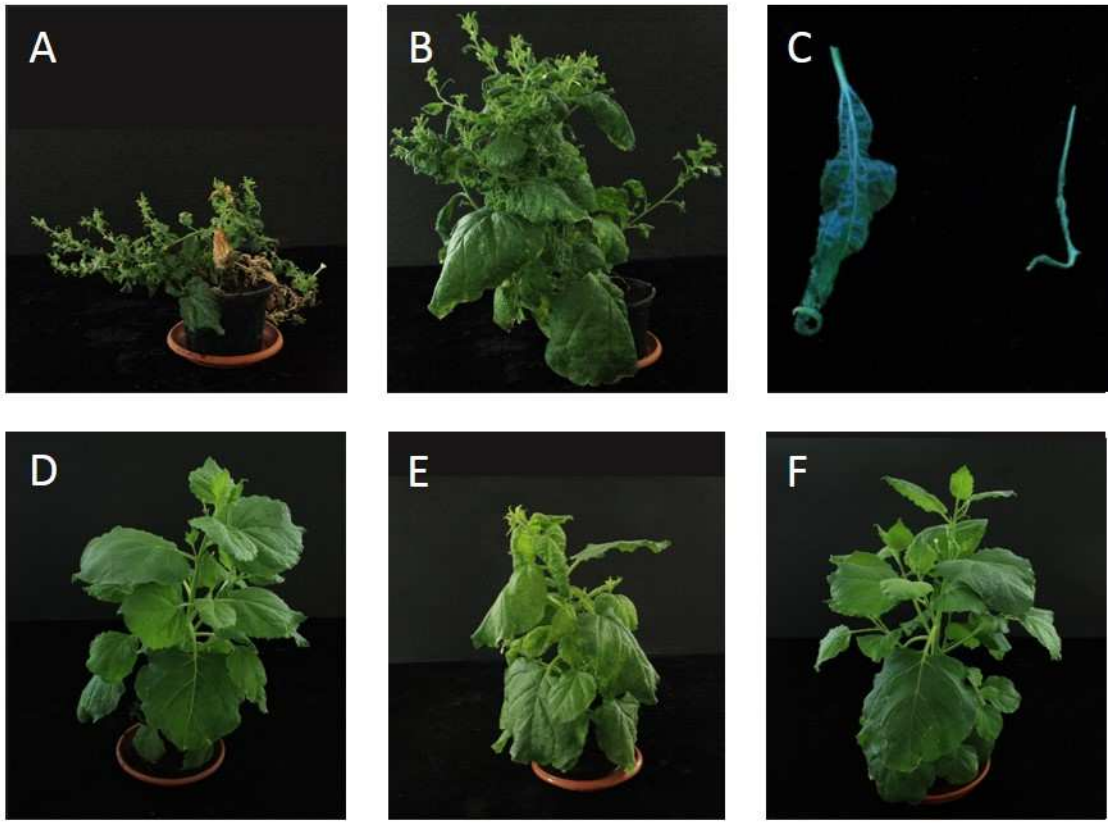

Fig. 3.1 *N. benthamiana* plants displaying symptoms of CMV infection with isolate PV-0506 causing stunted growth (**A**), isolate PV-0474 inducing leaf deformations (**B**) with shoestring leaves (**C**), symptomless isolate PV-0184 (**D**), isolate PV-0036 triggering severe chlorosis (**E**) and a healthy plant for comparison (**F**).

About one week after inoculation *N. benthamiana* plants developed symptoms in the case of 19 of the 29 CMV isolates from the DSMZ. The other ten isolates were symptomless, but the presence of viral RNA was verified by RT-PCR and gel electrophoresis (see Part 2.4). Four of the isolates were selected for further investigations, because they caused either severe symptoms in the case of the three isolates PV-0506, PV-0474 and PV-0036 or no symptoms (isolate PV-0184). Isolate PV-0506 of subgroup IB lead predominantly to stunted plant growth (Fig. 3.1A), isolate PV-0474 of subgroup IB triggered leaf deformations with shoestring leaves (Fig. 3.1B and C), isolate PV-0036 of subgroup IA induced severe chlorosis (Fig. 3.1E) and isolate PV-0184 of subgroup II was symptomless on infected *N. benthamiana* plants (Fig. 3.1D). RNAs of the four isolates were extracted, transcribed, amplified and successfully cloned into pDIVA. With *R. radiobacter* the cloned RNAs could be propagated and infiltrated in *N. benthamiana* plants. The cloned RNAs were part of the vector T-DNAs that were transferred by the bacteria into plant cells in which the viral genes were transiently expressed. When all three cloned RNAs of an isolate or of a pseudorecombinant were used together for infiltration, viral replication was induced in plant cells from the cloned and transferred RNAs with the help of transiently expressed viral proteins from these RNAs and host proteins. Infections were triggered by the three cloned RNAs of all four isolates confirmed by symptom development and/or RNA extraction and RT-PCR. So infectious full-length clones of the four isolates were created that consist of three pDIVA vectors for each isolate encoding its three genomic RNAs. These full-length clones induced the same symptoms on

N. benthamiana plants as the original isolates: stunted plant growth for the clone of PV-0506, leaf deformations with shoestring leaves for the clone of PV-0474, severe chlorosis for the clone of PV-0036 and no symptoms for the clone of PV-0184 (Fig. 3.1).

The original isolates and the full-length clones of PV-0506, PV-0474 and PV-0036 successfully infected the *C. sativus* cultivars Vorgebirgstrauben and Delikatess and caused mosaic symptoms (Fig. 3.2). Other cultivars like Bimbostar F1, Darina and Picolino were less successfully (PV-0506) or not at all (PV-0474 and PV-0036) systemically infected. Isolate and clone PV-0184 did not cause a systemic infection on any tested *C. sativus* cultivar. *N. benthamiana* plants had been inoculated at the same time and were tested positively for PV-0184 by RT-PCR and gel electrophoresis to confirm that the inoculation procedure worked.

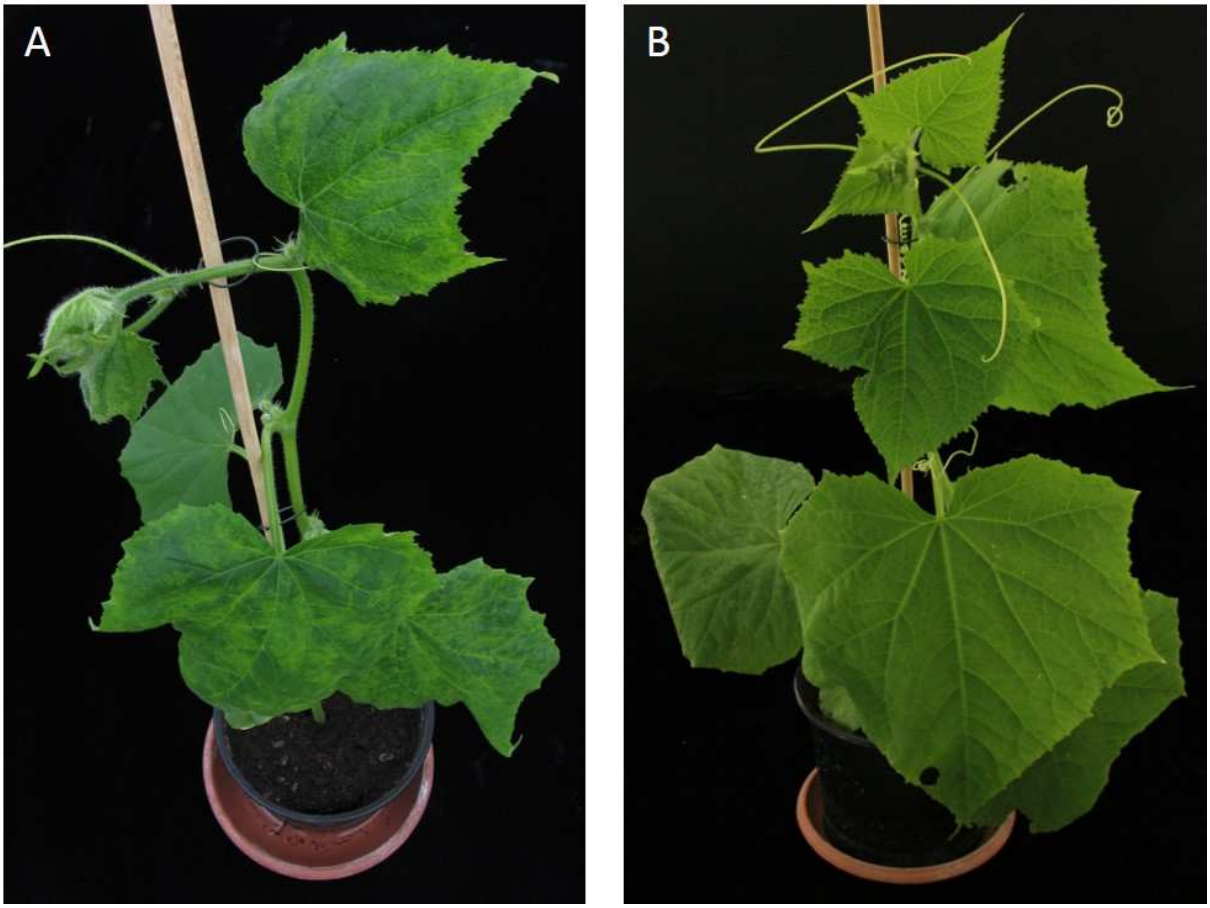

Fig. 3.2 Mosaic symptoms on *C. sativus* cultivar Vorgebirgstrauben induced by CMV full-length clone PV-0474 (**A**) and a healthy plant for comparison (**B**).

3.4.2 Sequencing of the cloned RNAs and phylogenetic analysis of the full-length clones

The complete sequences of all three RNAs of all four infectious full-length clones of the CMV isolates PV-0506, PV-0474, PV-0184 and PV-0036 were determined. This was achieved in a step wise approach for each RNA. The sequencing files were assembled to the full RNAs and sequences published in GenBank (Table 3.1). For the phylogenetic analysis of the isolates, alignments of each RNA of all four isolates, of reference CMV strains of the three subgroups and of *Peanut stunt virus* isolate PSV ER (accession number U15728) were created. Reference CMV strains for subgrouping were: Tfn for subgroup IB (accession number Y16924), Fny for subgroup IA (accession number NC_002034) and LS for subgroup II (accession number AF416899). From these alignments phylogenetic trees were created

with the software MEGA6 (Tamura et al., 2013) using the Neighbor-Joining method (Saitou and Nei, 1987) with 2,000 bootstrap replications (Felsenstein, 1985) and the evolutionary distances determined as number of base substitutions per site using the Maximum Composite Likelihood method (Tamura et al., 2004). PSV ER served as an outgroup. The classification of the four cloned isolates was the same according to the phylogenetic analysis of all three RNAs. The results for RNA1 are shown in Fig. 3.3, while the grouping looks the same for the other two RNAs with only the bootstrap and branch length values differing (data not shown). Isolates PV-0506 and PV-0474 belong to subgroup IB, isolate PV-0036 to subgroup IA and isolate PV-0184 to subgroup II and no reassortment of RNAs from different subgroups could be observed.

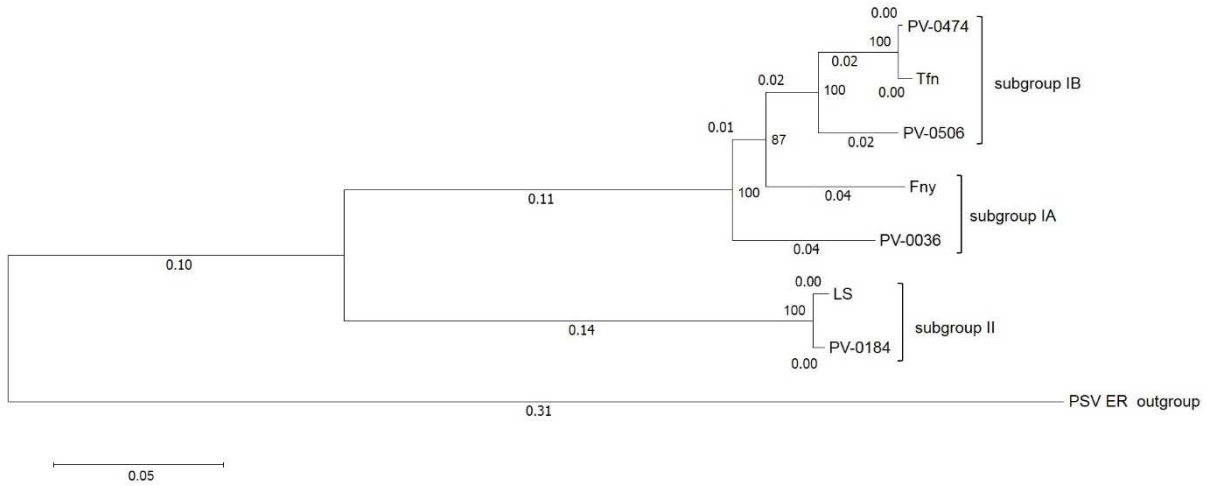

Fig. 3.3 Relationship of the four CMV full-length clones, reference strains of CMV and PSV ER as outgroup. Only the classification according to RNA1 is shown here, but for the other two RNAs the grouping looks the same with only the bootstrap and branch length values varying. The optimal tree with the sum of branch length = 0.83043244 was inferred using the Neighbor-Joining method in MEGA6. Bootstrap values (2,000 replicates) are shown next to the branches. The tree is drawn to scale and the evolutionary distances were computed as number of base substitutions per site using the Maximum Composite Likelihood method. There were a total of 3,242 positions in the final dataset with all positions containing gaps and missing data being eliminated.

3.4.3 Studies of pseudorecombinants of the cloned CMV isolates

Table 3.3 Pseudorecombinants of the four CMV isolates with their denotations, the backgrounds of their RNAs and the induced symptoms on *N. benthamiana* and *C. sativus*. Pseudorecombinant 144 was not tested on *C. sativus*.

Denotation	RNA1 background	RNA2 background	RNA3 background	Symptoms on *N. benthamiana*	Symptoms on *C. sativus*
511	PV-0506	PV-0184	PV-0184	Mosaic	Non
151	PV-0184	PV-0506	PV-0184	Mosaic	Non
115	PV-0184	PV-0184	PV-0506	Non	Mosaic
411	PV-0474	PV-0184	PV-0184	Deformation	Mosaic
141	PV-0184	PV-0474	PV-0184	Mosaic	Non
114	PV-0184	PV-0184	PV-0474	Mosaic	Mosaic
311	PV-0036	PV-0184	PV-0184	Chlorosis	Mosaic
131	PV-0184	PV-0036	PV-0184	Chlorosis	Mosaic
113	PV-0184	PV-0184	PV-0036	Chlorosis	Mosaic
144	PV-0184	PV-0474	PV-0474	Non	Not tested

As listed in Table 3.3 all pseudorecombinants infected *N. benthamiana* and *C. sativus* plants and most of them produced symptoms. On *N. benthamiana* only the pseudorecombinants 115 and 144 did not

induce symptoms, while on *C. sativus* the pseudorecombinants 511, 151 and 141 were symptomless. RNA of all pseudorecombinants was detected in plants of both species. For *N. benthamiana* almost all inoculated plants were infected with the pseudorecombinants, while the percentage of infected *C. sativus* plants varied for the different pseudorecombinants. Some infected almost all plants and others only a single plant. On *N. benthamiana* the pseudorecombinant 411 led to leaf deformations with shoestring leaves similar to those found for the original isolate and clone PV-0474. Severe chloroses similar to those found for the original isolate and clone PV-0036 were generated by the three pseudorecombinants 311, 131 and 113. On *C. sativus* mosaic symptoms of differing severity were caused. Pseudorecombinants 511 and 151 caused only mild mosaic symptoms on *N. benthamiana*. Pseudorecombinant 144 was only tested on *N. benthamiana* to further investigate the role of RNA1 for leaf deformations caused by isolate PV-0474 on this plant species.

3.4.4 Semi-quantitative ELISA

With the semi-quantitative ELISA, the virus titers were assessed. Values of virus-infected samples were significantly distinguishable from samples of healthy control plants. High titers were observed for isolate PV-0474, median titers for the pseudorecombinants 411 and 144 and low titers for PV-0184 (data not shown). This indicates that pseudorecombinants 411 and 144 possibly replicate to a higher extend in *N. benthamiana* plants than the isolate PV-0184 but to a lower extend than the isolate PV-0474.

3.5 Discussion

Infectious full-length clones of the four CMV isolates PV-0506, PV-0474, PV-0036 and PV-0184 were successfully established. Phylogenetic analyses of their nucleotide sequences revealed that each of them belongs to one of the three CMV subgroups (IA, IB and II). This classification was identical for all three RNAs, therefore, no reassortment and recombination events between RNAs of different subgroups were observed. Isolates PV-0506 and PV-0474 belong to subgroup IB, isolate PV-0036 to subgroup IA and isolate PV-0184 to subgroup II. CMV derived from the full-length clones induced either the same strong symptoms or was symptomless as the wild-type isolates on *N. benthamiana*. Clone and isolate PV-0506 led to stunted plant growth, clone and isolate PV-0474 to leaf deformations with shoestring leaves, clone and isolate PV-0036 to severe chlorosis and clone and isolate PV-0184 to no symptoms. This fits to the common notion in literature that subgroup II strains and isolates generally induce no or only mild symptoms (Mochizuki and Ohki, 2012). On the one hand, appearance and severity of symptoms depends on environmental conditions and the host plant, which were the same in our study. On the other hand, the virus specifics influence the symptomatology (Palukaitis et al., 1992). These include virus species, subgroup, strain, isolate and possible satellite RNAs, which can aggravate or attenuate the symptoms of their helper virus (Piazzolla et al., 1998). A role of satellite RNAs can be excluded in our case, because the infectious full-length clones did not contain a cloned satellite RNA and caused the same variation of symptoms as the original isolates.

Only two of the *C. sativus* cultivars were systemically infected by most of the isolates and clones. The cultivars Vorgebirgstrauben and Delikatess do not have resistances against CMV as our results show and non are stated by seed producers and retailers. The isolates PV-0506, PV-0474 and PV-0036 and their full-length clones induced mosaic symptoms, while the isolate PV-0184 and its full-length clone did not cause a systemic infection in these cultivars. The other three cultivars Darina, Bimbostar F1 and Picolino were not systemically infected by most of the tested isolates. These cultivars show efficient

resistances as described by producers and retailers. No symptoms were caused and no viral RNA was detected. Only isolate PV-0506 caused infections in single inoculated plants. It is a characteristic of CMV resistances that they only work against certain strains (Essafi et al., 2009). The complete genome analysis of *C. sativus* by Huang et al. (2009) revealed possible pathogen resistance genes. These encode either nucleotide-binding site leucine-rich repeat (NBS-LRR) proteins functioning as direct or indirect receptors for pathogens often triggering a hypersensitive response, proteins of the lipoxygenase (LOX) pathway producing aldehydes and alcohols involved in plant defense reactions or translation initiation factors of the eIF4E and eIF4G types which can prevent viruses from using the translation machinery if mutated (Essafi et al., 2009; Huang et al., 2009).

Pseudorecombinants of the four cloned isolates were created for an initial approach to localize the determinants for the symptomatology in *N. benthamiana* and *C. sativus*. All three RNAs have been found to determine symptoms caused by certain CMV strains on different host plants (Shintaku et al., 1992; Ding et al., 1994; Diveki et al., 2004; Phan et al., 2014). On *C. sativus* all nine tested pseudorecombinants were infectious, while on *N. benthamiana* also a tenth pseudorecombinant was tested and found infectious. All three combinations of PV-0036 and PV-0184 induced similar symptoms to those caused by PV-0036 on both plant species, chlorosis on *N. benthamiana* and mosaic symptoms on *C. sativus*. All pseudorecombinants caused strong symptoms except for pseudorecombinant 113, which induced only mild mosaic symptoms on *C. sativus*. Thus all three RNAs of this isolate play a role in inducing the chlorosis on *N. benthamiana* and the mosaic symptoms on *C. sativus*. A linkage between chlorosis induction on *Nicotiana* species and RNA3 of CMV was reported (Rao and Francki, 1982), which was further delimited to the CP gene (Shintaku and Palukaitis, 1990) and even confined to one specific amino acid at position 129 of the CP (Shintaku, 1991). Mosaic-inducing strains have a proline at this position, while it is occupied by a serine or leucine in chlorosis-inducing strains. Site-directed mutagenesis to change leucine to proline changed the phenotype from chlorotic to mosaic and vice versa (Shintaku et al., 1992). Isolate PV-0036 inducing chlorosis also contains a leucine at this position, while the other three isolates causing different or no symptoms at all have a proline there. But chlorosis was also caused by the two pseudorecombinants 311 and 131, which have the CP of PV-0184 with a proline at position 129. So likely the amino acid at position 129 of the CP is not solely responsible for the chlorosis phenotype in all cases. The pseudorecombinants 511 and 151 induced only mild mosaic symptoms on *N. benthamiana* and no symptoms on *C. sativus*, while 115 caused no symptoms on *N. benthamiana* but mosaic symptoms on *C. sativus*. In this case a determinant for the stunted growth phenotype displayed by PV-0506 infected *N. benthamiana* could not be further delimited. It seems like two to three RNAs of this isolate and/or their gene products have to be present for the development of this severe phenotype, while RNA1 or RNA2 alone is responsible for mosaic symptoms on *N. benthamiana*. The determinant for the mosaic symptoms on *C. sativus* is located on RNA3. All pseudorecombinants of isolates PV-0474 and PV-0184 infected *N. benthamiana* with symptoms, 411 caused strong symptoms, while 141 and 114 induced only mild mosaic symptoms. On *C. sativus* pseudorecombinant 141 was symptomless, while 114 caused mild and 411 strong mosaic symptoms. The determinant for mosaic symptoms could be located to RNA1 and RNA3 for *C. sativus* and to RNA2 and RNA3 for *N. benthamiana*. Zhang et al. (1994) localized the symptom determinant for severe, systemic mosaic symptoms on tobacco on RNA1 and RNA2 of subgroup I isolate CMV Fny by pseudorecombination experiments. The targeting of miRNA-regulated plant development and the inhibition of the plant Argonaute (AGO) defense proteins in RNA-induced silencing complexes (RISC)

by the 2b protein of CMV was described to determine viral symptoms in *Arabidopsis thaliana* (Lewsey et al., 2007; Zhang et al., 1994). Additionally, Phan et al. (2014) identified RNA2 as the symptom determinant for leaf distortion and stunting in *N. benthamiana* induced by isolate CMV Fny through pseudorecombination experiments. Our results do not indicate that the 2b protein on RNA2 has a particular effect on the symptomatology of the isolates in *N. benthamiana* and *C. sativus*.

Pseudorecombinant 411 induced the same severe symptoms of shoestring-like deformed leaves on *N. benthamiana* as the original isolate PV-0474 and its clone. So the determinant for this phenotype could be localized on RNA1 of PV-0474. On this RNA, more specifically at amino acid position 461 of the encoded 1a protein, the determinant for necrotic lesions induced by the CMV isolate Ns on *Nicotiana* spp. is localized (Diveki et al., 2004). Isolate Ns contains a cysteine and non-necrotic isolates an arginine at this position. Site-directed mutagenesis to change cysteine to arginine prevented the necrotic phenotype of isolate Ns, while mutagenesis from arginine to cysteine induced the necrotic phenotype (Diveki et al., 2004). This shows that RNA1 can contain a symptom determinant. However, all of the investigated isolates in this study contained an arginine at the described position and none induced necrotic lesions on *N. benthamiana*.

Because RNA1 encodes the 1a protein involved in replication, a higher virus titer could be the cause of the severe symptoms of isolate PV-0474. To clarify this, the virus titer was determined with a semi-quantitative ELISA. The results suggested a slightly increased virus titer for the pseudorecombinants 411 and 144 compared to isolate PV-0184 possibly being caused by a slightly higher replication rate. The pseudorecombinants 411 and 144 showed similar virus titers by similar ELISA readings, although 144 did not cause symptoms while 411 induced strong symptoms. This disproves that the replication capacity and virus titer are causative for the severe symptoms induced by the isolate PV-0474. Other characteristics of RNA1, its encoded 1a protein and their interactions with plant metabolism are responsible for the leaf deformations on *N. benthamiana*. A comparison of the 1a proteins of the four isolates revealed a number of amino acid substitutions between them. The 1a protein of PV-0474 and PV-0506 is very similar only differing in 16 from 994 amino acids. Isolates PV-0474 and PV-0036 differ in 28 amino acids of the 1a protein while isolates PV-0474 and PV-0184 have 148 different amino acids in their 1a protein. The amino acid substitutions are evenly distributed over the 1a protein. Some of 16 amino acids differing between the 1a protein of PV-0506 and PV-0474 seem to be responsible for the severe symptoms and could be investigated by further experiments. The established infectious full-length clones of isolates from different subgroups with varying symptomatology are suited for further characterization experiments of CMV.

4 Development of a molecular assay for the general detection of tospoviruses and the distinction between tospoviral species

Niklas Bald-Blume[1], Jan Bergervoet[2], Edgar Maiss[1]

[1]Department of Phytomedicine, Institute of Horticultural Production Systems, Leibniz Universität Hannover, Hannover, Germany
[2]Plant Sciences Group, Wageningen University and Research Centre, Wageningen, Netherlands

4.1 Abstract

A Luminex xTAG based assay for plant infecting tospoviruses was developed. The test enables the detection of tospoviruses in general and the differentiation of the four important member species of this genus: *Tomato spotted wilt virus*, *Impatiens necrotic spot virus*, Capsicum chlorosis virus and *Watermelon silver mottle virus*. The generic *Tospovirus* primers used in this method are furthermore applicable in a basic RT-PCR detection of tospoviruses and an economic alternative based on a restriction fragment length polymorphism (RFLP) is described for the distinction of the four mentioned and additional member viruses. The sophisticated Luminex xTAG technology allows the simultaneous detection of various targets. This study is part of a project to develop a simultaneous detection method for various plant pathogens (viral, bacterial and fungal) in plant material.

4.2 Introduction

The genus *Tospovirus* comprises all plant infecting viruses of the family *Bunyaviridae*. The other four genera of this family (*Orthobunyavirus*, *Phlebovirus*, *Nairovirus* and *Hantavirus*) contain animal infecting viruses. The genus *Tospovirus* obtained its name from the type species *Tomato spotted wilt virus* (TSWV) and contains 11 approved species and 20 tentative species (see Table 1.1 in Part 1) (Hassani-Mehraban et al., 2016; International Committee on Taxonomy of Viruses, 2015; Plyusnin et al., 2012). These viruses have quasi-spherical particles with a diameter of 80-120 nm enveloped by a host-derived membrane. The two glycoproteins Gn and Gc are embedded in this membrane. Particles contain a tripartite single-stranded RNA genome with negative- or ambisense polarity. The three RNAs differ in size and are thus called large (L), medium (M) and small (S). All three RNAs are incorporated in one particle but independently packaged by many copies of the nucleoprotein and a few copies of the viral RNA-dependent RNA polymerase (Kormelink et al., 2011; Turina et al., 2012). Tospoviruses have a large host range, TSWV infects for example 1,090 plant species in 85 families containing many economically important crop plants as well as numerous weed species (Parrella et al., 2003), while other tospoviral species have a more restricted host range. Some of the members are found worldwide like TSWV, *Impatiens necrotic spot virus* (INSV) and *Iris yellow spot virus* (IYSV), while others are restricted to certain regions like *Watermelon silver mottle virus* (WSMoV) to Asia, Capsicum chlorosis virus (CaCV) to Asia and Australasia and *Polygonum ringspot virus* (PolRSV) to Europe (Pappu et al., 2009). The same applies to the thrips vector species (insect order: *Thysanoptera*), some have a worldwide distribution like *Frankliniella occidentalis* (Kirk and Terry, 2003), while others are restricted to single countries like *Thrips setosus* to Japan (Murai, 2001). Both species are vectors of TSWV (Rotenberg et al., 2015). In total fifteen species of thrips transmit tospoviruses in a persistent and propagative manner (Rotenberg et al., 2015) and are critical for their epidemiology (Pappu et al., 2009).

For the established *Tospovirus* species host ranges and geographical distributions have increased in recent years. Additionally, new species belonging to this genus have been described. Because of these points and the provoked loss of produce and economic damage, tospoviruses are counted to the most devastating plant viruses (Rybicki, 2015; Scholthof et al., 2011). This makes the diagnosis of tospoviral infections an important issue, especially for plant protection services trying to confine further spread of associated diseases. ELISA, RT-PCR and multiplex RT-PCR can be used to detect tospoviruses and to discriminate between its species. Because of its robustness and sensitivity, ELISA is often the standard method of choice for plant virus diagnostics (Voller et al., 1976). Unfortunately, the results are not always clear and *Tospovirus*-specific antibodies often show cross reactivity among species of this genus making ELISA based diagnosis difficult. Polyclonal antibodies against the nucleocapsid protein of WSMoV also interact with *Groundnut bud necrosis virus* (GBNV), *Watermelon bud necrosis virus* (WBNV) and CaCV (Mandal et al., 2012). The same cross reactivity is also described by commercial antibody or ELISA test suppliers like the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (DSMZ; Germany), Agdia (USA) and LOEWE Biochemica (Germany). For TSWV antibodies possible cross reactions with *Groundnut ringspot virus* (GRSV), *Tomato chlorotic spot virus* (TCSV) and Chrysanthemum stem necrosis virus (CSNV) are known. This can complicate distinction of tospoviral species by ELISA. Additionally, an ELISA test has to be performed independently for every species, since multiplexing is not possible. Multiplex RT-PCRs can be a solution for this and have been described for the detection of TSWV, INSV, CSNV, IYSV and CaCV (Kuwabara et al., 2010) or TSWV, Melon yellow spot virus (MYSV), WSMoV, INSV and IYSV (Uga and Tsuda, 2005).

In this study a new method for plant virus diagnosis is described using the Luminex xTAG technology to test for tospoviruses in general and for the five species TSWV, WSMoV, INSV, IYSV and CaCV. Virus samples of 12 *Tospovirus* isolates from eight different species were obtained from the DSMZ. The nucleic acid based assay platform of the Luminex xTAG technology allows the simultaneous detection of theoretically up to 500 analytes in one sample. Tospoviral RNA is transcribed into cDNA and amplified in a first RT-PCR. The products are then subjected to a target specific primer extension (TSPE) reaction for which tagged primers and biotinylated dCTP are used. The primer tags allow the hybridization of TSPE products to complementary anti-tags coupled to Luminex MagPlex-TAG Microspheres. These paramagnetic polystyrene "beads" are filled with a mix of two to three fluorescent dyes at different ratios enabling their later identification through excitation and measured fluorescence and thus the identification of bound TSPE products. The presence of hybridized TSPE products is revealed through the binding of streptavidin-R-phycoerythrin to the incorporated biotin, its excitation and detection of the resulting fluorescence. The test procedure is described in detail by van der Vlugt et al. (2015). This technique has been used in human medicine for diagnosis of respiratory viruses like influenza- and coronaviruses amongst others (Mahony et al., 2007). In recent years it has been adapted for the diagnostics of plant pathogens. Van Brunschot et al. detected and distinguished different begomoviruses and their whitefly vectors (2014a) and different pospiviroids (2014b) applying this method. Lim et al. (2016) used it for the simultaneous detection of three lily-infecting viruses already demonstrating the advantage of this approach as tests for certain pathogens could be combined in the future to cover the most important diseases of a crop. Our work should contribute to this end as part of a project for the development of a simultaneous detection method for various viral, bacterial and fungal plant pathogens.

Additionally, this study describes a more economic approach for generic *Tospovirus* detection by RT-PCR and for differentiation of tospoviral species by restriction fragment length polymorphism (RFLP).

4.3 Materials and Methods

4.3.1 Virus specimens

Infected, dried plant material of 12 tospoviral isolates from eight different species was ordered from the DSMZ. From Alstroemeria necrotic streak virus (ANSV), CaCV, GRSV, IYSV, TCSV and WSMoV one isolate each was received, while three isolates were obtained from INSV and TSWV. Information about the viral origin (host plant, country of origin and provider) was supplied by the DSMZ (Table 4.1) for most isolates. All 12 isolates were mechanically inoculated on *Nicotiana benthamiana* plants using phosphate buffer (0.05 M KH_2PO_4, 0.05 M Na_2HPO_4, 1 mM EDTA, 5 mM Na-DIECA) with a spatula tip of celite and charcoal. Inoculated plants were grown in a greenhouse and monitored for symptom expression. Leaves of plants showing systemic symptoms (after one to two weeks) were used for RNA extraction.

Table 4.1 *Tospovirus* species and isolates from the DSMZ with information about the original host plant, the country of origin and the provider as supplied by the DSMZ.

Tospovirus species	Isolate	Host plant	Country of origin	Provider
Alstroemeria necrotic streak virus (ANSV)	PV-1027	*Alstroemeria sp.*	Colombia	R. Kormelink
Capsicum chlorosis virus (CaCV)	PV-0864	*Solanum lycopersicum*	Thailand	E. Maiss
Groundnut ringspot virus (GRSV)	PV-0205	*Arachis hypogaea*	South Africa	G. Pietersen
Impatiens necrotic spot virus (INSV)	PV-0280	*Hippeastrum sp.*	USA	O.W. Barnett
INSV	PV-0281	*Anemone coronaria*	Germany	J. Dalchow
INSV	PV-0485	*Gloxinia sp.*	unknown	P. Roggero
Iris yellow spot virus (IYSV)	PV-0528	*Allium ampeloprasum*	unknown	J.T.J. Verhoeven
Tomato chlorotic spot virus (TCSV)	PV-0391	*Capsicum annuum*	Brazil	O. Lovisolo
Tomato spotted wilt virus (TSWV)	PV-0182	*Nicotiana tabacum*	Bulgaria	M. Jankulova
TSWV	PV-0204	*Impatiens New Guinea Hybrid*	unknown	D.-E. Lesemann
TSWV	PV-0393	*Nicotiana tabacum*	Bulgaria	M. Jankulova
Watermelon silver mottle virus (WSMoV)	PV-0283	*Solanum lycopersicum*	Taiwan	S.K. Green

4.3.2 Nucleic acid extraction

Total RNA was extracted once from infected, dried plant material, twice from infected *N. benthamiana* plants and three times from healthy *N. benthamiana* plants using the RNeasy Plant Mini Kit (Qiagen, Germany) following the manufacturer's instructions.

4.3.3 Primer design

Primers for general tospovirus detection, for pre-amplification and for TSPE reactions were designed on the basis of alignments of nucleotide sequences. Sequences of segment M were retrieved from the GenBank of the National Center for Biotechnology Information (NCBI), imported into CLC Main Workbench (CLC bio, Denmark) and aligned. For the general detection primers Tospo_GENs/as, the pre-amplification primers Tospo_OUTs/Tospo_GENas and the generic TSPE primers tTospo_GENs/as the alignments were analyzed for conserved regions and corresponding sequences were chosen for the

primers. Some degenerations were inserted into the primer sequences. For the species specific TSPE primers tTSWVs/as, tINSVs/as, tWSMoVs/as, tCaCVs/as and tIYSVs/as conserved regions for each of the species were identified in the alignments and relevant sequences used for the primers. The pre-amplification primers (Nad5s/as) for the NADH dehydrogenase subunit 5 gene (*nad5*) from Menzel et al. (2002) and TSPE primer (tNad5) for *nad5* from van Brunschot et al. (2014a) adapted from Botermans et al. (2013) were used as internal control primers. The tag sequences were attributed to all TSPE primers by Luminex (USA) complementary to anti-tags on the microspheres' surfaces. The primers were synthesized by Eurofins Scientific (Luxembourg). Table 4.2 displays their characteristics.

Table 4.2 Characteristics of oligonucleotide primers used in this study. TSPE primer names are preceded by a t, their tag sequences are italicized and their corresponding microspheres are listed.

Name	Sequence (5'-3')	Length [nts]	Fragment size [bp]	Corresponding microsphere
Tospo_GENs	TCHTNCCAACHTGGRAYAG	19	420	
Tospo_GENas	TGCADGCYTCAATNAADGC	19	480	
Tospo_OUTs	TVACHAAYTGGAARAATGA	19		
Nad5s	GATGCTTCTTGGGGCTTCTTGTT	23	180	
Nad5as	CTCCAGTCACCAACATTGGCATAA	24		
tTospo_GENs	*CTTAAACTCTACTTACTTCTAATT*-TCHTNCCAACHTGGRAYAG	43	420	MTAG-A056
tTospo_GENas	*CATAAATCTTCTCATTCTAACAAA*-TGCADGCYTCAATNAADGC	43		MTAG-A075
tTSWVs	*ACAAATATCTAACTACTATCACAA*-AACCCTACAGGGAAAC	40	180	MTAG-A039
tTSWVas	*TTAACAACTTATACAAACACAAAC*-CTGCACATCAAATGC	39		MTAG-A053
tINSVs	*ATACTTTACAAACAAATAACACAC*-ACCAAGATAATTAAGATACA	44	260	MTAG-A019
tINSVas	*ATCTCAATTACAATAACACACAAA*-AAGCTGAACACAATTC	40		MTAG-A067
tWSMoVs	*CACTACACATTTATCATAACAAAT*-GTCAGTTTCACTATAAGC	42	260	MTAG-A042
tWSMoVas	*CTATCATTTATCTCTTTCTCAATT*-TAAGTTGCATGCACTG	40		MTAG-A072
tCaCVs	*CTAAATCACATACTTAACAACAAA*-GTCAGCTTCACTATAAAT	42	260	MTAG-A063
tCaCVas	*TTCAATTCAAATCAAACACATCAT*-TTGAGTTGCATGCAGTA	41		MTAG-A064
tIYSVs	*AATTTCTTCTCTTTCTTTCACAAT*-AGCTGTACCATCAATATTATA	45	260	MTAG-A014
tIYSVas	*TCATCACTTTCTTTACTTTACATT*-TTAAGCTGCATACATCT	41		MTAG-A044
tNad5	*AACTTTCTCTCTCTATTCTTATTT*-AGGATCCGCATAGCCCTCGATTTATGTG	52		MTAG-A043

4.3.4 Pre-amplification RT-PCR

Viral RNA was transcribed into cDNA and then amplified using the Access RT-PCR System kit (Promega, USA) applying the concentrations specified by the manufacturer in 25 µl reactions in covered 96-well Multiply PCR plates (Sarstedt, Germany). The following incubations were carried out: 45 min at 45 °C, 2 min at 94 °C, 30 cycles of 30 s at 94 °C, 30 s at 47 °C and 30 s at 68 °C and a final extension for 7 min at 68 °C. The internal control primers (Nad5s/as) and the degenerate primers (Tospo_OUTs/Tospo_GENas) were used, which enabled the production of a 180 bp fragment for all plant samples and a 480 bp fragment for all tospoviruses. After RT-PCR 5 µl of each product was stained

with 1 µl loading buffer (37.5 % glycerol, 0.2 % bromophenol blue, 125X GelRed (Biotium, USA)) and loaded on an agarose gel (1 %) to check for the expected fragments.

4.3.5 TSPE Reaction

The pre-amplification products were directly used for a multiplex TSPE reaction. Primers are extended and Biotin-14-dCTP (Thermo Fisher Scientific, USA) was incorporated instead of normal dCTP with the remaining unmodified nucleotides (dATP, dGTP, dTTP; Thermo Fisher Scientific). A set of 13 primers was used, two generic primers to detect all tospoviruses (tTospo_GENs/as), ten specific primers to identify five tospovirus species (tTSWVs/as, tINSVs/as, tWSMoVs/as, tCaCVs/as and tIYSVs/as) and one plant internal control primer (tNad5) for *nad5*. TSPE mixes of 20 µl containing 5 µl of the pre-amplification products were prepared (0.75 U Platinum GenoType Tsp DNA Polymerase (Thermo Fisher Scientific), 5 µM Biotin-14-dCTP, 5 µM eachof normal dATP, dGTP and dTTP, 1.5 mM $MgCl_2$, 25 nM of each primer, 1X PCR Rxn Buffer (Thermo Fisher Scientific), sterile water up to 20 µl). The reaction mix was incubated under the following conditions in covered 96-well Multiply PCR plates: One cycle for 2 min at 94 °C, 30 cycles of 0.5 min at 94 °C, 0.5 min at 45 °C and 1 min at 72 °C and a final extension for 5 min at 72 °C.

4.3.6 Microsphere hybridization

The TSPE products were hybridized to the corresponding beads. A mix of the 13 Luminex MagPlex-TAG Microspheres MTAG-A075, MTAG-A072, MTAG-A067, MTAG-A064, MTAG-A063, MTAG-A056, MTAG-A053, MTAG-A044, MTAG-A043, MTAG-A042, MTAG-A039, MTAG-A019 and MTAG-A014 (0.5 µl of each bead per reaction containing 1,250 beads) was prepared in 2X Tm hybridization buffer (0.4 M NaCl, 0.2 M Tris, 0.16 % Triton X-100, pH 8.0; 25 µl per reaction) and double distilled and deionized water (13.5 µl per reaction). The mixture was split into 96-well polycarbonate microplates (Corning, USA) and 5 µl of the TSPE products were added to each well for a final volume of 50 µl with 25 microspheres each per µl hybridization mixture. Interactions between the complementary tag sequences of the TSPE primers and anti-tag sequences coupled to the microspheres allowed the hybridization of TSPE products to the beads. To facilitate this the mixtures were first denatured at 96 °C for 90 s and then incubated at 37 °C for 30 min.

4.3.7 Luminex assay

After hybridization the mixtures were moved to Cellstar 96-well cell culture plates (Greiner Bio-One, Austria) and the microspheres were pelleted on a magnetic separator for 1 min. The supernatants were discarded and the beads resuspended and washed two times in 80 µl 1X Tm hybridization buffer per well. The supernatants were removed again and 80 µl of 1X Tm hybridization buffer containing streptavidin-R-phycoerythrin (2 µg/ml; Thermo Fisher Scientific) were added to each well. The plates were protected from light and incubated on a shaker (600 rpm) at room temperature for 15 min. After pelleting the microspheres again, the supernatants were discarded and the microspheres resuspended in 90 µl 1X Tm hybridization buffer per well. Finally, 70 µl per sample were analyzed in a Luminex 200 System with the xPONENT Software (Version 3.0; Luminex). A red laser (635 nm) excited the bead dyes and a green laser (532 nm) the R-phycoerythrin bound via streptavidin and biotin to the TSPE products. The fluorescence of the R-phycoerythrin was recorded as the median fluorescence intensity (MFI) signal allowing positive or negative evaluation for the presence of amplified plant or tospoviral nucleic acids in the samples. Identification of these nucleic acids could be achieved by the fluorescence

of the bead dyes which are unique for each microsphere with its specific anti-tag. Three independent measurements were performed with the three RNA extractions described above.

4.3.8 RT-PCR and RFLP

For the general detection of tospoviruses RNA from infected plant material was transcribed into cDNA by incubating 4 µl water, 2 µl RNA, 1 µl Primer Tospo_GENas (10 µM) and 0.5 µl dNTP mix (10 mM each) at 99 °C for 3 min, cooling the reaction mix on ice, adding 0.5 µl RevertAid Reverse Transcriptase (20 U/µl) and 2 µl 5X (RevertAid) Reaction Buffer (both from Thermo Fisher Scientific) and incubating the mixture at 42 °C for 60 min. The cDNA was then amplified in a PCR. The PCR reaction mix consisted of 10 µl Phusion Flash High-Fidelity PCR Master Mix (Thermo Fisher Scientific), 6 µl water, 2 µl cDNA, 1 µl Primer Tospo_GENs and 1 µl Primer Tospo_GENas and was incubated under the following conditions: One cycle for 15 s at 98 °C, 34 cycles of 5 s at 98 °C, 5 s at 52 °C and 15 s at 72 °C and a final extension for 5 min at 72 °C. PCR products were analyzed by gel electrophoresis on an agarose gel (1 %) after adding 4 µl loading buffer. For distinction of tospoviruses by RFLP an RT-PCR was performed like above and the products were digested with the restriction enzymes *Hinf*I (Thermo Fisher Scientific) and *Bcl*I (New England Biolabs, USA). For that 20 µl of each PCR product were mixed with 2 µl *Hinf*I (10 U/µl), 2 µl *Bcl*I (10 U/µl), 3 µl 10X FastDigest Buffer (Thermo Fisher Scientific) and 3 µl water and incubated at 37 °C for one hour. After that 6 µl loading buffer were added to each reaction and the products were analyzed by gel electrophoresis on an agarose gel (3 %).

4.4 Results

4.4.1 Luminex assay for *Tospovirus* detection and distinction

In conventional RT-PCR experiments it was verified that the pre-amplification and generic primers worked with *Tospovirus* RNA in general and that the species specific primers were specific for the corresponding virus species (data not shown). After pre-amplifications with the four primers Tospo_OUTs/Tospo_GENas and Nad5s/as the RT-PCR products were used in TSPE reactions with all 13 TSPE primers (tTospo_GENs/as, tTSWVs/as, tINSVs/as, tWSMoVs/as, tCaCVs/as, tIYSVs/as and tNad5). TSPE products were hybridized to the corresponding 13 MagPlex-TAG Microspheres (MTAG-A056, MTAG-A075, MTAG-A039, MTAG-A053, MTAG-A019, MTAG-A067, MTAG-A042, MTAG-A072, MTAG-A063, MTAG-A064, MTAG-A014, MTAG-A044 and MTAG-A043) and the hybridization mix was then analyzed in three independent Luminex tests each with their own RNA extractions, pre-amplifications, TSPE reactions, hybridizations and Luminex measurements. Mean values of the MFIs of three independent measurements were determined and plotted in Fig. 4.1. The generic primer tTospo_GENs together with bead MTAG-A056 lead to the detection of most tospoviruses except IYSV and WSMoV. The positive signal ranged from 333 for one INSV isolate to 1,135 for one TSWV isolate. IYSV and WSMoV infected plant material produced a signal of 117 respectively 123 not exceeding the threshold set at 323 (light grey columns in Fig. 4.1). The internal control TSPE primer tNad5 worked with MTAG-A043 and lead to the detection of plant material in all samples with MFIs ranging from 415 for one INSV isolate to 1,048 for CaCV. Only in the WSMoV samples the threshold was not reached with a value of 239 (checkered columns in Fig. 4.1). All species specific sense primers worked well with their corresponding microspheres. They allowed the detection of the species they were designed for and discriminated all other tospovirus species. Only the IYSV specific TSPE primers tIYSVs/as did not generate satisfactory results in the Luminex test together with

beads MTAG-A014 and MTAG-A044 (data not shown). The tCaCVs primer together with MTAG-A063 induced a high signal of 1,499 in the CaCV infected samples but in no other sample and control (black columns in Fig. 4.1). The tINSVs primer lead to high MFI values of 1,148 to 1,708 only in the plant material infected with the three INSV isolates together with MTAG-A019 (diagonally striped columns in Fig. 4.1). The signal induced by the tWSMoVs primer with MTAG-A042 was slightly lower with 594 but also restricted to plants infected with this virus (white columns in Fig. 4.1). The MFI values generated by the tTSWVs primer with MTAG-A039 were also slightly lower with 455 to 891 and restricted to samples containing the three TSWV isolates tested. In this case a slight reaction (MFI of 268) also occurred in plants infected with ANSV (dark grey columns in Fig. 4.1). The remaining five antisense TSPE primers (tTospo_GENas, tTSWVas, tINSVas, tWSMoVas and tCaCVas) together with the residual five microspheres (MTAG-A075, MTAG-A053, MTAG-A067, MTAG-A072 and MTAG-A064) did not generate satisfactory results in Luminex tests for tospovirus detection and distinction (data not shown). Nevertheless, this was not necessary, as one primer is sufficient for the TSPE reaction and the following Luminex test. As a threshold for all tests two times the MFI value of the water control from the tNad5 test was selected, because this sample showed the highest MFI value of all healthy plant and water controls. The values of the other tests were comparable, but were left out of the figure for the sake of clarity (dashed line in Fig. 4.1).

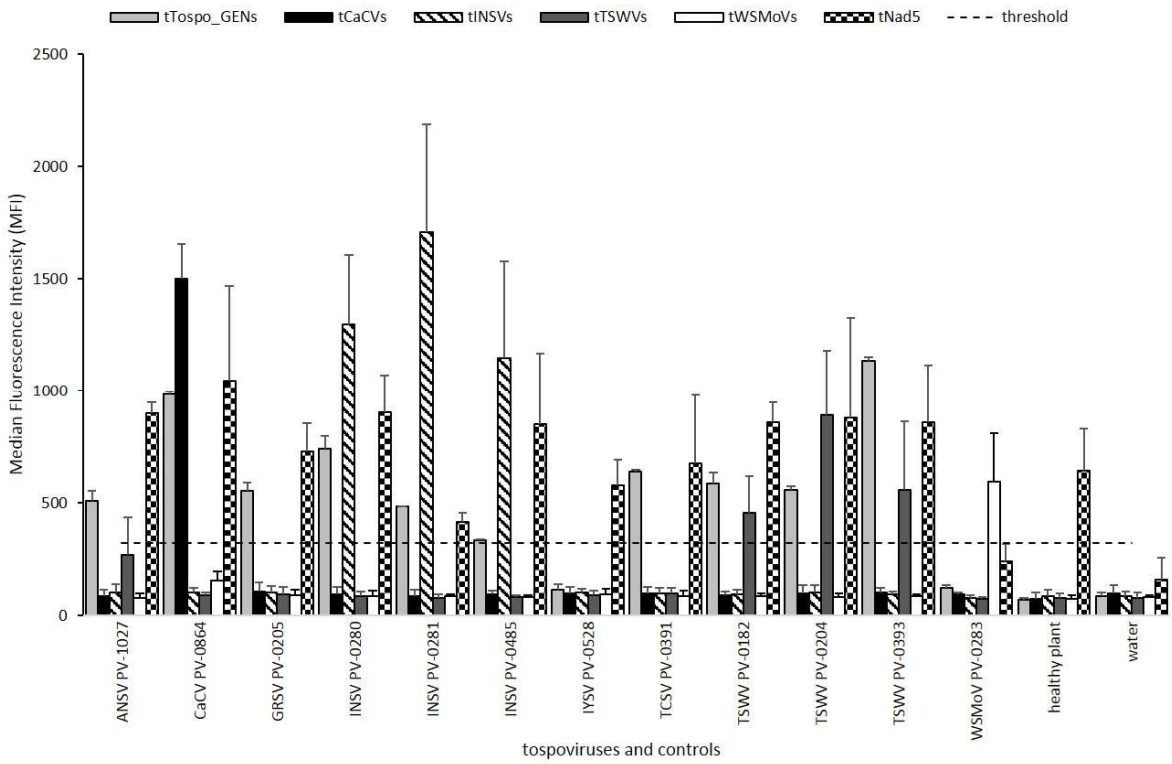

Fig. 4.1 Results of three independent measurements of the Luminex assay. The tests were conducted against tospoviruses in general, against the tospoviral species CaCV, INSV, TSWV and WSMoV as well as against a plant internal control. Plant material isolated from a series of plants each infected with 1 of the 12 tospoviral isolates, healthy plant material and a water control were applied in each test. The MFI values were measured and the mean values of the three measurements were plotted as well as the standard deviations. The threshold for all tests was set at two times the mean MFI value of the water control of the tNad5 test (dashed line).

4.4.2 Generic and specific *Tospovirus* detection by RT-PCR

The generic *Tospovirus* primers Tospo_GENs/as (without tags) allowed the detection of all eight tospoviruses and of all three isolates of INSV and TSWV tested in RT-PCR experiments. The expected fragment of about 420 bp was produced when using RNA extracted from infected plants. The primers generated no band when using RNA from healthy plants or from plants infected with other viruses like *Cucumber mosaic virus* (CMV), *Plum pox virus* (PPV) and *Pepper mild mottle virus* (PMMoV) (Fig. 4.2) demonstrating the specificity of the primers.

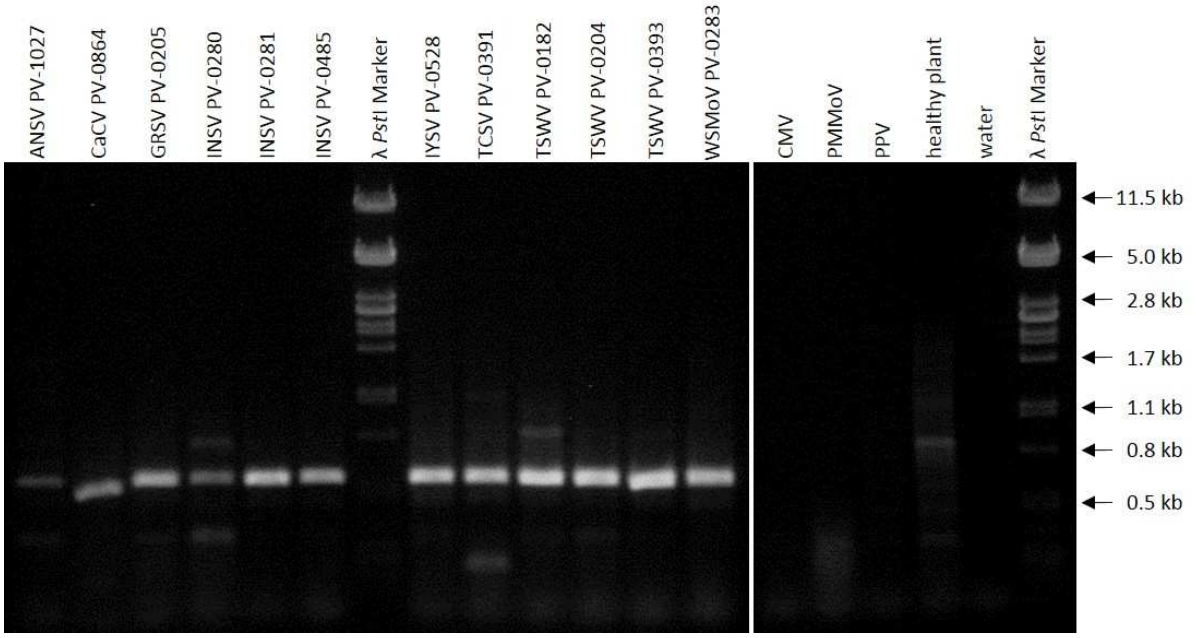

Fig. 4.2 Results of a RT-PCR with RNA from plants infected with 12 *Tospovirus* isolates, CMV, PMMoV and PPV as well as RNA from healthy plants and a water control using primers Tospo_GENs/as after gel electrophoresis. As molecular-weight size marker *Enterobacteria phage λ* DNA digested with *Pst*I was used. DNA fragments appear larger than the expected 420 bp, because the DNA stain GelRed changes the migration speed of DNA depending on the concentrations of DNA and GelRed (Huang et al., 2010).

The five species specific primer pairs (without tags) also worked in normal RT-PCRs for the identification of these species (data not shown). They lead to the production of the expected fragments only when RNA from plant material infected with the corresponding virus was used. For TSWV the specific fragment was 180 bp in size and for CaCV, INSV, IYSV and WSMoV 260 bp. Primer sequences can be deduced from Table 4.2 omitting the tag sequences in italics.

4.4.3 RFLP distinction of tospoviruses

Most of the examined tospovirus species could be distinguished by a RFLP analysis using the restriction enzymes *Hinf*I and *Bcl*I after RT-PCR with primers Tospo_GENs/as. The PCR products of the tospoviruses have different restriction enzyme recognition sites which leads to a distinct pattern for most tospoviruses after gel electrophoresis. RFLP analysis was first performed *in silico* using the software CLC Main Workbench and tospoviral segment M sequences from the GenBank to predict fragment sizes. After RT-PCR, digestion and RFLP analysis the predicted fragments were observed for most viruses, for TSWV these were 325 and 90 bp, for INSV 260, 90 and 70 bp, for WSMoV 290, 50, 30 and 20 bp, for CaCV 280, 80 and 30 bp, for GRSV 320 and 90 bp, for TCSV 140, 90 and 50 bp and for IYSV 310, 80 and 20 bp and additionally a predicted but not detected 10 bp fragment. For ANSV no

predictions could be made *in silico*, because for this virus only a partial segment S sequence with the nucleocapsid protein gene is available. Nevertheless, its RFLP pattern was determined and fragments of about 320 and 90 bp were detected. So its pattern was similar to that of GRSV and TSWV. These three viruses could not be clearly differentiated. CaCV, INSV, IYSV, TCSV and WSMoV could be distinguished from these viruses and from one another (Fig. 4.3).

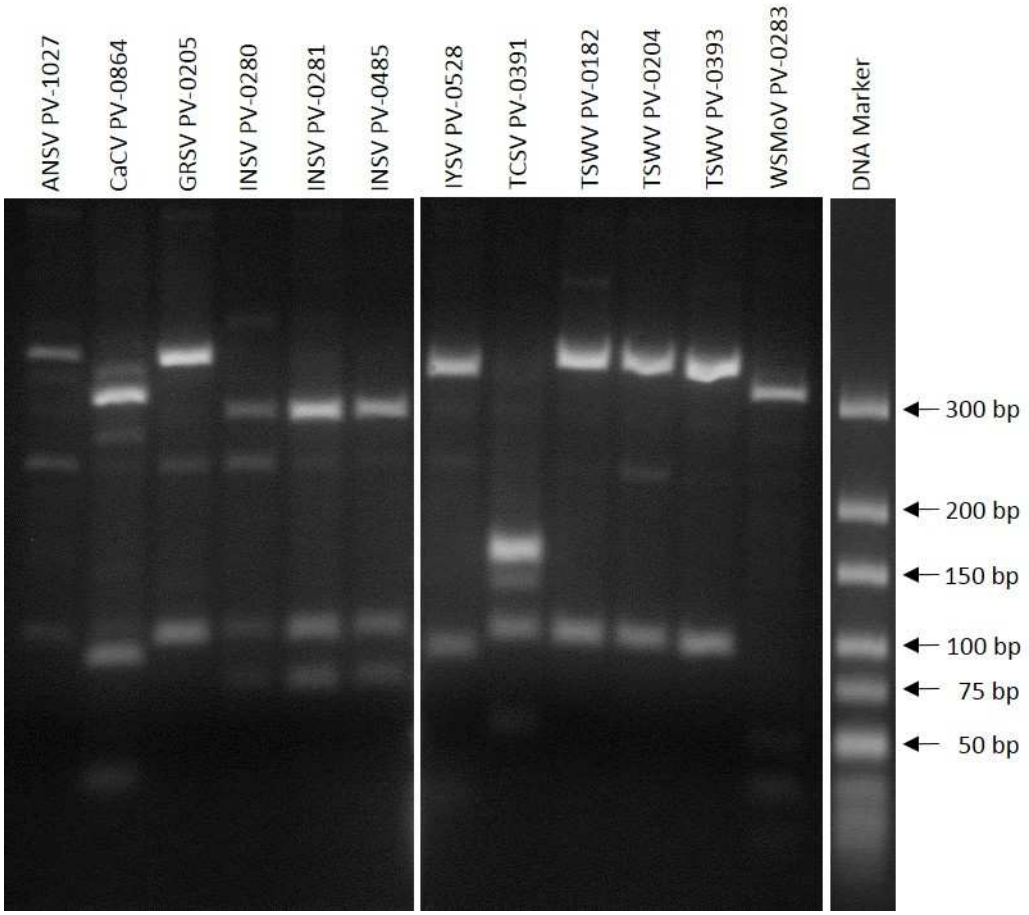

Fig. 4.3 Results of a RFLP after gel electrophoresis. RNA from plants infected with 12 *Tospovirus* isolates was transcribed and amplified in a RT-PCR with primers Tospo_GENs/as. PCR products were then digested using *Hinf*I and *Bcl*I. The O'GeneRuler Ultra Low Range DNA Ladder (Thermo Fisher Scientific) was used as molecular-weight size marker. The figure was assembled from two gels.

4.5 Discussion

A molecular assay for the detection of tospoviruses in general and for the four species TSWV, INSV, CaCV and WSMoV belonging to this genus using the Luminex xTAG technology was successfully developed. The generic *Tospovirus* test with primer tTospo_GENs detected six of the eight tested species. It failed to prove the presence of IYSV and WSMoV. However, the same primer without its tag in combination with an antisense primer detected these two viruses in RT-PCR experiments. So with some adaptations in the nucleotide sequence of the primer tTospo_GENs the generic Luminex test should also be able to cover these two species. The species specific tests applying the primers tCaCVs, tINSVs, tTSWVs and tWSMoVs were specific for these viruses. In the case of tINSVs and tTSWVs all three isolates of each of these two viruses were successfully detected. A test for IYSV was not established.

This study is one of only a few employing the Luminex xTAG technology for the detection of plant pathogens and the first for tospoviruses. So far it has been applied to screen for begomoviruses and pospiviroids (van Brunschot et al., 2014a; van Brunschot et al., 2014b), for CMV, *Lily mottle virus* (LMoV) and *Lily symptomless virus* (LSV) in lily plants (Lim et al., 2016) and for CMV and its two subgroups (see Part 2). The related Luminex xMAP technology utilizing coupled antibodies instead of oligonucleotides has been used to identify *Potato virus X* (PVX), *Potato virus Y* (PVY) and *Potato leaf roll virus* (PLRV) (Bergervoet et al., 2008) as well as *Plum pox virus* (PPV) (Croft et al., 2008) in plant material. But because of cross reactivity between different tospoviral species described for antibodies by researchers (Hassani-Mehraban et al., 2016; Mandal et al., 2012) and antisera suppliers (DSMZ, Agdia and LOEWE Biochemica), which might be a problem in the antibody based Luminex xMAP test for the differentiation of *Tospovirus* species, a nucleic acid based array was thought to be advantageous. An advantage of both these assay formats is their multiplexing capability and combined with this the potential for the simultaneous detection of various diseases in plant samples. The study of Lim et al. (2016) is an example for this since they used a Luminex xTAG assay for the detection of the three viruses CMV, LMoV and LSV infecting lily plants. The standard method for virus detection, the ELISA, lacks this potential and is quite labor and time intensive as it only allows to test for one virus at a time. Charlermroj et al. (2014) have created a multiplex antibody array similar to an ELISA for the simultaneous detection of the three viruses MYSV, WSMoV and *Chilli veinal mottle virus* (ChiVMV) as well as the fruit blotch bacterium *Acidovorax avenae* subspecies *citrulli*. All four pathogens were immobilized by capture antibodies specific to the four pathogens in each well at preassigned positions, detected by fluorescently conjugated secondary antibodies and identified by their position in the microwells. But this procedure is far from routine application. A mixed detection method combining RT-PCR and ELISA was developed and applied for the detection of the four tospoviruses CaCV, MYSV, *Tomato necrotic ringspot virus* (TNRV) and WSMoV first transcribing and amplifying RNA by RT-PCR using degenerate primers and digoxigenin (DIG) labelled dUTP, then hybridizing PCR products to four species-specific biotinylated probes in streptavidin-coated microtiter wells and finally detecting the labelled and hybridized PCR products by ELISA using a peroxidase-conjugated anti-DIG antibody (Charoenvilaisiri et al., 2014).

Our Luminex xTAG test for some tospoviruses could be combined with already existing and prospective tests for plant pathogens to create assays for certain crops identifying their most important diseases similar to the respiratory virus panel test of Mahony et al. (2007) that screens for 20 different human respiratory viruses and their subtypes. For example, for tomato crops (*Solanum lycopersicum*) the tests for TSWV, CaCV and INSV from this study, for *Tomato yellow leaf curl virus* (TYLCV) from van Brunschot et al. (2014a), for *Tomato apical stunt viroid* (TASVd), *Tomato chlorotic dwarf viroid* (TCDVd) and *Tomato planta macho viroid* (TPMVd) from van Brunschot et al. (2014b) and for CMV from Lim et al. (2016) or from us (see Part 2) could be combined. Further tests could be added to cover the most important of the 136 viruses infecting tomatoes (Brunt et al., 1997; Hanssen et al., 2010) and also bacterial and fungal pathogens.

A more economical alternative for generic *Tospovirus* detection and species distinction was successfully developed in this study. The primer Tospo_GENs/as detected RNA of all eight examined tospoviruses in RT-PCRs but not of viruses from other genera. This is one of the best coverages of tospoviral species obtained by one primer pair so far (see Table 4 in Hassani-Mehraban et al., 2016). Chen et al. (2012) designed two degenerate primer pairs that transcribed and amplified RNA of 12 species. One of the pairs

binds to segment L and the other to the NSm gene of segment M. Their antisense primer gM870c for segment M in part coincides with our Tospo_GENas primer. Since our primer is more degenerated, our primers might also identify RNA from the additional tospoviral species GBNV, Calla lily chlorotic spot virus (CCSV), MYSV, WBNV and Tomato yellow ring virus (TYRV) tested by Chen et al. (2012) but not by us. Hassani-Mehraban et al. (2016) describe six primer pairs and RT-PCRs that cover 20 assigned and tentative tospoviral species and classify them into six subgroups.

Also the five species specific primer pairs (without tags) can be used in RT-PCRs for the identification of these species. They lead to the amplification of nucleic acids only of the corresponding species and of all three isolates tested from either TSWV or INSV. They cannot or only in part be used for multiplex RT-PCRs, because the CaCV, INSV, IYSV and WSMoV specific primers lead to fragments of the same size. So a distinction of these species would not be possible by a plain multiplex RT-PCR. The fragments of the generic *Tospovirus* primers and the TSWV specific primers can be distinguished from these primers and from one another, though, and could be combined with one of the other four primer pairs for a clear multiplex RT-PCR. Kuwabara et al. (2010) described such a multiplex RT-PCR for the detection of the five tospoviruses TSWV, INSV, CSNV, IYSV and CaCV.

Alternatively, RT-PCR products of the different *Tospovirus* species generated with the generic primers Tospo_GENs/as could be differentiated by RFLP using the restriction enzymes *Hinf*I and *Bcl*I. The five species CaCV, IYSV, INSV, TCSV and WSMoV were clearly distinguished. The additional three species ANSV, GRSV and TSWV could be discriminated from the rest but not clearly from each other. Chu et al. (2001) used this method to detect and differentiate the five tospoviruses TSWV, GRSV, INSV, WSMoV and *Peanut chlorotic fan-spot virus* (PCFV) using primers binding to segment L and the restriction enzyme *Xba*I.

Three approaches of varying complexity and costs have been described for the successful detection and differentiation of various tospoviruses species.

5 General Discussion

In this study a Luminex xTAG test for *Cucumber mosaic virus* (CMV) and its subgroups I and II was successfully established. It reliably detected all tested CMV isolates and could differentiate almost all isolates by their subgroup. Only one isolate (PV-0185) could not be classified into a subgroup with this technique. The subgrouping was confirmed by a standard subgroup specific ELISA, which could not classify the two isolates PV-0185 and PV-0189. Therefore, the Luminex test outclassed the ELISA in the field of subgroup differentiation. That a fraction of CMV isolates evade detection by subgroup specific antisera has already been reported (Hsu et al., 2000). Similarly a few isolates of hepatitis B virus (HBV) were not detected by an ELISA with seven monoclonal antibodies raised against the six genotypes of HBV (Usuda et al., 1999). Evasion of detection is caused by differences in one or more of the identified epitopes for monoclonal antibodies (Hsu et al., 2000; Usuda et al., 1999). One of the ten epitopes identified for CMV could contain amino acid 13 of the CMV coat protein (CP), because the two isolates evading ELISA detection have an amino acid substitution of serine at this position compared to an asparagine in the remaining isolates. This amino acid substitution could also render both isolates symptomless. Conspicuously, they are the only subgroup I isolates not causing symptoms. Milder or no symptoms at all are usually attributed to subgroup II isolates (Carrère et al, 1999). Unexpectedly isolate PV-0185 evaded the subgroup specific Luminex detection, although the virus-specific part of the TSPE primer tCMV_SGIas corresponded completely to the isolate's sequence. Additionally, both subgroup I specific primers (tCMV_SGIs/as) even with attached tag sequences led to the production of the expected fragment in normal RT-PCRs. This might be due to a problem with the tag of primer tCMV_SGIas interacting unspecifically with CMV or plant sequences. A search with the Basic Local Alignment Search Tool for nucleotides (blastn) (http://blast.ncbi.nlm.nih.gov/Blast.cgi) of the NCBI revealed a maximal congruence of 12 nucleotides between the tag and CMV sequences and 11 nucleotides between the tag and *N benthamiana* sequences from 24 possible nucleotides of the tag. This can imply that the tagged primers might bind incorrectly prohibiting correct amplification. A solution for this putative problem could be a higher annealing temperature in the target specific primer extension (TSPE) reaction leading to more specific binding of primer tCMV_SGIas. However, an increased annealing temperature can be problematic because it might prohibit binding of the other primers (tCMV_GENas, tCMV_SGIIas and tNad5) in the multiplex TSPE reaction. Alternatively, the tag sequence of the tCMV_SGIas primer could be changed and concurrently the associated bead with the complementary anti-tag.

Detection of tospoviruses was also successful with the developed Luminex xTAG assay. The generic test reliably detected six of the eight species. It failed to cover the species *Watermelon silver mottle virus* (WSMoV) and *Iris yellow spot virus* (IYSV), although the generic primers without tag sequences detected both viruses in normal RT-PCRs. This might also be caused by unspecific interaction between the tag of primer tTospo_GENs with WSMoV, IYSV or plant sequences. A blastn search revealed a maximal congruence of 13 nucleotides between tag and *N. benthamiana*, 11 between tag and WSMoV and 10 between tag and IYSV sequences from 24 possible nucleotides of the tag. So the same problem and solutions as described above for the detection of CMV PV-0185 might apply to the detection of IYSV and WSMov. Alternatively, the degenerate virus-specific primer part might not fit well to both viruses' sequences leading to much lower amplification of these sequences compared to the other tospoviruses. Fewer products could hybridize to corresponding microspheres preventing high median

fluorescence intensity (MFI) values during measurements. Adaptation of the primer sequence including the position of degenerations or additional degenerations might lead to coverage of both viruses. Jin and Mattes (2011) modified degenerate qPCR primers for an improved quantification and comparison of functional genes of aerobic bacteria. Special software has been programmed for the design of degenerate primers like DPPrimer (Gahoi et al., 2013), Hyden (Linhart and Shamir, 2007) and CODEHOP (Staheli et al., 2011) and could be applied for adaptations of the tTospo_GENs primer. Increase of the annealing temperature, exchange of primer tag sequence and/or adaptation of primer degenerations could be evaluated for inclusion of WSMoV and IYSV in the generic test. Afterwards alterations of the annealing temperature or a modified primer could be tested with further *Tospovirus* species and isolates to assess their coverage by the old or new primers.

The species specific tests reliably detected and differentiated *Tomato spotted wilt virus* (TSWV), Capsicum chlorosis virus (CaCV), *Impatiens necrotic spot virus* (INSV) and WSMoV. The additional tospoviruses Alstroemeria necrotic streak virus (ANSV), *Tomato chlorotic spot virus* (TCSV) and *Groundnut ringspot virus* (GRSV) did not react in any of the specific tests. A specific test for IYSV was not established, although the IYSV specific primers without tag sequences worked in RT-PCRs and specifically amplified a IYSV fragment. This and similar findings concerning sequence congruence might again indicate a problem with the tag of the primers interacting unspecifically with IYSV or plant sequences as described above for the detection of CMV PV-0185. The same problem and solutions as above might also apply to the specific detection of IYSV.

A general lesson from the failed detection of single viruses or isolates is that the assignment of tags to the primers needs to be optimized to reduce sequence congruence between the tags and non-target sequences preventing correct binding of primers and thus amplification. This would involve more bioinformatic analyses. Recently a tool for the attribution of tags to primers for Luminex xTAG based assays has been introduced into the primer design software PrimerPlex and AlleleID (PREMIER Biosoft, USA), which might be helpful to reduce unwanted sequence congruence. Available virus sequences and full genome sequences for example of *N. benthamiana* (Bombarely et al., 2012), *C. sativus* (Huang et al., 2009) and *S. lycopersicum* (The Tomato Genome Consortium, 2012) can be consulted to reduce sequence congruence.

The results of this study and recent publications show that Luminex xTAG tests can be used for a generic detection of a group of viruses (e.g. a genus) as well as for the distinction of species or even subgroups of a species. The specificity of the test depends on the primers and can be influenced by primer adaptations. This is a big advantage compared to immunoassays like ELISA, lateral flow test (LFT) and Luminex xMAP. The specificity of antibodies can only be influenced to a limited extent. Luminex xTAG assays can also be applied for a multiplex detection of different targets like viruses and other pathogens at the same time. This is also an advantage over many other detection methods like ELISA, LFT and isothermal amplification methods. The *Tospovirus* xTAG test is a multiplex assay for six different targets: tospoviruses in general, TSWV, CaCV, INSV, WSMoV and plant Nad5 as an internal control. Conventional PCRs or RT-PCRs can be performed in a multiplex format. The number of targets is limited by the resolution of gel electrophoresis and the design of different sized amplicons. Uga and Tsuda (2005) developed a multiplex RT-PCR for the simultaneous detection of the five tospoviruses INSV, IYSV, Melon yellow spot virus (MYSV), TSWV and WSMoV. The five foodborne human pathogenic bacteria *Salmonella enterica*, *Staphylococcus aureus*, *Shigella flexneri*, *Listeria monocytogenes* and *E. coli* (Chen et al., 2012) and six different pathogenic *Listeria* species (Ryu et al.,

2013) were detected by a multiplex PCR. This seems to be the limit of distinguishable targets. The limitations of different sized amplicons and gel electrophoresis can be circumvented by using different fluorophore-labeled oligonucleotides (primers, molecular beacons, TaqMan probes etc.) in qPCR or RT-qPCR. Hu et al. (2014) used a molecular beacon based multiplex qPCR for the detection of the eight foodborne bacterial pathogens *S. enterica*, *L. monocytogenes*, *E. coli*, *Vibrio parahaemolyticus*, *Vibrio vulnificus*, *Campylobacter jejuni*, *Enterobacter sakazakii* and *Shigella* spp. A lot of different reporter dyes and a number of quenchers exist to label primers or probes. The number of detection channels that allow differentiation of reporter signals is limiting in thermal cyclers. Limiting factors for all multiplex methods based on amplification of nucleic acids, including PCR, qPCR and xTAG, are the design of multiplex primers. They should have similar annealing temperatures, should not be complementary to each other to prevent primer-dimers and should not have sequences similar to the other targets to inhibit unspecific amplification.

Until now, the developed xTAG assays for CMV and tospoviruses were tested individually and only with single infections. However, this already caused three positive signals in most cases: of the internal control, of the generic *Tospovirus* or CMV test and of the species or subgroup specific test. For further tests the two assays could be combined and also mixed infections could be investigated, which have been described for tospoviruses causing reassortment between species (Tentchev et al., 2011; Tripathi et al., 2015; Webster et al., 2011).

In the long run Luminex xTAG tests for different viruses and other plant pathogens could be merged to cover the most important pathogens for certain crops. Seed potatoes (*Solanum tuberosum*) for example are required to be free of at least the following 12 pathogens: *Potato spindle tuber viroid* (PSTVd), *Clavibacter michiganensis* subspecies *sepedonicus*, *Ralstonia solanacearum*, *Erwinia spp.*, *Pectobacterium spp.*, *Dickeya spp.*, *Potato virus X* (PVX), *Potato virus Y* (PVY), *Potato virus S* (PVS), *Potato virus M* (PVM), *Potato virus A* (PVA) and *Potato leafroll virus* (PLRV) (UNECE, 2014). An ELISA screening for all these pathogens individually is labor-intensive. So multiplexing would reduce the workload enormously. Charlermroj et al. (2014) developed a multiplex immunoassay similar to ELISA for the simultaneous detection of three viruses and one bacterium. However, this method is far from routine application and requires more elaborate equipment than a standard ELISA reader for the detection of fluorescently labeled antibodies at specific positions in microwells. A test for 12 different targets would exceed the number of detection channels of most thermal cyclers for multiplex qPCR. The xTAG technology would be able to cope with this number of simultaneous tests as the respiratory virus panel test for 20 human respiratory viruses and their subtypes has demonstrated (Mahony et al., 2007). It would also allow the differentiation of mild strains like PVY^{N} from severe strains like PVY^{NTN}, which are indistinguishable by ELISA. In addition to the pathogens listed above, a high number of further pathogens cause diseases in potato including *Potato mop-top virus* (PMTV), *Pseudomonas* spp., *Streptomyces* spp., *Alternaria* spp., *Fusarium* spp., *Phytophthora* spp. and *Rhizoctonia solani* among others (Hardy, 1996). More of these pathogens could be included in only one assay. In theory the Luminex xTAG technology would allow the simultaneous detection and distinction of 500 targets with the supplied 500 distinct microspheres and their specific anti-tags. But in practice this will not be realizable, because of the described multiplexing limits of RT-PCRs concerning annealing temperature, cross interactions and unspecific amplification. Also the test volume is restricted and will not allow such a high number of beads and associated primers.

An even greater multiplexing capacity is possible with the nucleic acid spot hybridization (NASH) microarrays, which allow the analysis of up to several thousand targets (Severgnini et al., 2011). The 12 foodborne human pathogenic bacteria *V. parahaemolyticus*, *Vibrio fluvialis*, *C. jejuni*, *Proteus* spp., *Yersinia enterocolitica*, *L. monocytogenes*, *E. coli*, *Shigella* spp., *Salmonella* spp., *Enterococcus faecalis*, *S. aureus* and *Streptococcus* spp. were identified and differentiated with this method (Huang et al., 2014). Microarrays are described as slower and less sensitive than other methods. They furthermore lack the potential for high throughput testing (Boonham et al., 2007). Additionally, complicated and inflexible preparation of biochips are required as well as expensive laboratory equipment for analysis. A combination of these factors has prevented laboratories from adopting microarrays for routine diagnostics.

For tomato (*Solanum lycopersicum*) the highest number of combinable xTAG tests already exists. From this work the assay could include the tests for tospoviruses in general, for the *Tospovirus* species TSWV, INSV and CaCV as well as for CMV. A generic begomovirus test, a specific Tomato yellow leaf curl virus (TYLCV) test and tests for the pospiviroids *Tomato apical stunt viroid* (TASVd), *Tomato chlorotic dwarf viroid* (TCDVd) and *Tomato planta macho viroid* (TPMVd) could be added from van Brunschot et al. (2014a; 2014b). Further developed tests could cover some of the 136 viruses infecting tomato (Hanssen et al., 2010) and bacteria like *C. michiganensis*, *Pseudomonas syringae*, *Xanthomonas campestris*, *Pectobacterium carotovorum* and *R. solanacearum* as well as fungi and oomycetes like *Alternaria* spp., *Fusarium oxysporum*, *Pythium* spp., *Phytophthora* spp. and *R. solani* among others (Paret et al., 2015).

The Luminex xTAG technology is connected with relatively high costs for equipment and chemicals (Luminex instruments, MagPlex-Tag Microspheres and enzymes), which are comparable to those of multiplex qPCR. The multiplexing potential of the xTAG technology can greatly reduce time- and labor-input though. So this technology can be interesting, especially for high throughput testing in routine laboratories.

Nevertheless, the generic primers for CMV and tospoviruses as well as the CMV subgroup and *Tospovirus* species specific primers of this study (without their tags) were proven to function in normal RT-PCRs. The CMV generic and subgroup specific primers covered all 29 tested CMV isolates. They can be used for reliably CMV detection and subgroup distinction. The generic *Tospovirus* primers detected all eight investigated *Tospovirus* species TSWV, CaCV, INSV, IYSV, WSMoV, ANSV, TCSV and GRSV. For INSV and TSWV three isolates each were covered. This is one of the best coverages of tospoviral species achieved by a single primer pair (compare with Table 4 in Hassani-Mehraban et al., 2016). Chen et al. (2012) developed two primer pairs covering 12 species. One of their primers (gM870c) overlaps with the Tospo_GENas primer of this study. As the Tospo_GENas primer is more degenerated it possibly also covers the tospoviral species *Groundnut bud necrosis virus* (GBNV), Calla lily chlorotic spot virus (CCSV), MYSV, *Watermelon bud necrosis virus* (WBNV) and Tomato yellow ring virus (TYRV) that are included in amplification with the gM870c primer by Chen et al. (2012). It could be investigated if RNA of these and further species is amplified by the generic primer pair of this study. The five specific primer pairs for TSWV, CaCV, INSV, IYSV and WSMoV reliably worked for these single species. Three isolates each of INSV and TSWV were covered. Further isolates of the five species could be tested to check if they are also included. The species specific primers cannot be applied in multiplex RT-PCRs, because the CaCV, INSV, IYSV and WSMoV specific primers amplify fragments of the same size. Only the fragments of the TSWV specific primers and of the generic

Tospovirus primers can be distinguished from these amplicons. For a multiplex RT-PCR of the five tospoviruses TSWV, INSV, IYSV, CaCV and Chrysanthemum stem necrosis virus (CSNV) the reader is referred to Kuwabara et al. (2010).

This study also describes a RFLP analysis for distinction of *Tospovirus* species. Tospoviral RNA is first transcribed and amplified in an RT-PCR with primers Tospo_GENs/as and then digested with the restriction enzymes *Hinf*I and *Bcl*I leading to distinguishable fragments for the five species CaCV, IYSV, INSV, TCSV and WSMoV. The three species ANSV, GRSV and TSWV have similarly sized fragments and can be differentiated from the other five tospoviruses but not from each other. Similarly, the five tospoviruses TSWV, GRSV, INSV, WSMoV and Peanut chlorotic fan-spot virus (PCFV) have been distinguished by RFLP using primers binding to segment L and the restriction enzyme *Xba*I (Chu et al., 2001).

In addition to the developed detection method for CMV and tospoviruses based on the Luminex xTAG technology, this study further characterized CMV. Twenty-nine isolates obtained from the DSMZ were investigated. All infected *Nicotiana benthamiana* plants. Their subgrouping was determined by a subgroup specific ELISA, by sequencing of the CP gene and by the subgroup specific Luminex test. The CP genes were also cloned into the pGEM-T Easy vector for further experiments. The ELISA and the Luminex test allowed classification into subgroup I and II, while the CP gene sequences enabled a further classification of subgroup I isolates into subgroup IA and IB. According to all three methods the same nine isolates belonged to subgroup II and 20 isolates to subgroup I. The Luminex test failed to classify one isolate and the ELISA two isolates belonging to subgroup I according to the CP gene sequences. Of the subgroup I isolates nine could be further classified into subgroup IA and eleven into subgroup IB. Most of the subgroup I isolates caused symptoms on *N. benthamiana* except for the two isolates PV-0185 and PV-0189 that also did not react in the ELISA. Both isolates have an amino acid substitution in their CP that might render them symptomless and might prevent binding of the subgroup I specific antibodies (see above). Most of the subgroup II isolates were symptomless except for isolate PV-0418, which was inconspicuous apart from causing symptoms on *N. benthamiana*. The subgroup II strains and isolates are generally regarded to be less virulent and to cause milder symptoms (Carrère et al., 1999; Mochizuki and Ohki, 2012). Subgrouping of the isolates according to all three methods is incomplete though, as they only act on the CP gene located on RNA3 or on the encoded CP protein. The three RNAs of CMV can have independent evolutionary histories generated by reassortment or recombination between CMV strains and also with other *Cucumovirus* species (Bonnet et al., 2005; Nouri et al., 2014; Roossinck, 2002; White et al., 1995). This implies that RNA1, RNA2 and even parts of RNA3 can be from different subgroups than the CP gene. The complete genome consisting of the three RNAs would have to be sequenced to determine the subgroup origin of all RNAs. But serology and CP gene sequences are generally consulted for subgrouping (Rasoulpour et al., 2016; Kumar et al., 2015).

Four of the isolates belonging to all three subgroups according to the methods described above and causing different severe symptoms on *N. benthamiana* were further investigated. The isolate PV-0184 belonging to subgroup II was infectious without producing symptoms, while the other three isolates generated strong symptoms: PV-0506 of subgroup IB stunted growth, PV-0474 of subgroup IB shoestring-like deformed leaves and PV-0036 of subgroup IA severe chlorosis. Full-length clones of these four isolates were created and the sequences of their genomic RNAs determined. Phylogenetic analysis of sequence data confirmed the subgrouping according to CP gene sequences alone. So no

reassortment and recombination events between subgroups were observed for these isolates. The full-length clones were infectious in *N. benthamiana* and either induced the same symptoms or were symptomless as the original isolates. So an effect of satellite RNAs aggravating or attenuating the symptomatology (Piazzolla et al., 1998) of the four isolates can be excluded. As the environmental conditions and the host plants were the same for the isolates, the different symptomatologies are determined by their genomic RNAs. All three RNAs have been described to determine the symptomatology of CMV strains on different host plants (Shintaku et al., 1992; Ding et al., 1994; Diveki et al., 2004).

Pseudorecombinants of the four full-length clones were generated to localize the determinants for the different symptoms caused by the isolates on *N. benthamiana*. They are denoted with a three number code after the isolate background of their RNA1 (1st number), RNA2 (2nd number) and RNA3 (3rd number) with "1" standing for isolate PV-0184, "3" for PV-0036, "4" for PV-0474 and "5" for PV-0506. As an example the pseudorecombinant named 511 consisted of RNA1 from PV-0506 and both RNA2 as well as RNA3 from PV-0184 (see Part 3 Table 3.3). All established pseudorecombinants were infectious. The three combinations containing either RNA1, RNA2 or RNA3 of PV-0036 and the remaining two RNAs of PV-0184 induced severe chlorosis just like isolate PV-0036. So all three RNAs of this isolate seem to trigger these symptoms. This contradicts to earlier findings that a single amino acid at position 129 of the CP encoded on RNA3 is responsible for chlorosis in *Nicotiana* spp. (Shintaku et al., 1992; Shintaku, 1991). The three combinations of PV-0506 and PV-0184 induced only mild mosaic symptoms (511 and 151) or no symptoms at all (115) on *N. benthamiana*. The stunted growth phenotype was not induced. So not a single RNA of PV-0506 is responsible for it, but a combination of two or even all three RNAs. Further pseudorecombinants could be generated containing two RNAs of PV-0506 and only one RNA of PV-0184 to investigate this. Only mild mosaic symptoms were caused by the two combinations 141 and 114 of PV-0474 and PV-0184 RNAs. Like isolate PV-0474 the pseudorecombinant 411 induced strong symptoms of shoestring-like deformed leaves on *N. benthamiana* implying that this phenotype is determined by RNA1 of PV-0474. Diveki et al. (2004) have localized the determinant for necrotic lesion symptoms caused by CMV Ns isolate on *Nicotiana* spp. to amino acid position 461 of the 1a protein supporting the findings of this study. A semi-quantitative ELISA comparing virus titers of the different isolates and pseudorecombinants excluded the higher virus titer alone from being the cause for the severe symptoms of isolate PV-0474. The pseudorecombinants 411 and 144 had similar ELISA readings, which means that their virus titers were comparable. Pseudorecombinant 411 caused severe symptoms while pseudorecombinant 144 was symptomless. So a higher replication rate induced by a more efficient 1a protein of PV-0474 leading to higher virus titers was not causing the severe symptoms of this isolate and pseudorecombinant 411. Other characteristics of RNA1, its encoded 1a protein and their interactions with plant metabolism determine these strong symptoms. A number of amino acid substitutions between the 1a proteins of the isolates were identified by alignments and pairwise comparisons. The 1a protein of PV-0474 and PV-0506 is very similar only differing in 16 from 994 amino acids. Isolates PV-0474 and PV-0036 differ in 28 amino acids of the 1a protein while isolates PV-0474 and PV-0184 have 148 different amino acids in their 1a protein. The amino acid substitutions are evenly distributed over the 1a protein. There are no regions with clustered substitutions. Some of 16 amino acids differing between the 1a protein of PV-0506 and PV-0474 seem to be responsible for the leaf deformations caused by PV-0474 on *N. benthamiana* and could be investigated in further experiments.

The full-length clones and pseudorecombinants of the four isolates were also tested on the additional host plant *Cucumis sativus*. Five cultivars of this plant species were investigated. Because the three cultivars Darina, Bimbostar F1 and Picolino showed efficient resistances and only single plants were systemically infected by isolate PV-0506 alone, the two remaining susceptible cultivars Vorgebirgstrauben and Delikatess were used for further experiments. The three isolates PV-0506, PV-0474 and PV-0036 caused mosaic symptoms, while isolate PV-0184 was not systemically infectious. All created pseudorecombinants of the four full-length clones were systemically infectious. All combinations of PV-0036 and PV-0184 induced mosaic symptoms. So all three RNAs of PV-0036 can trigger these symptoms. For isolate PV-0506 the determinant for mosaic symptoms was located on RNA3, because only the pseudorecombinant 115 induced these symptoms while the other two pseudorecombinants were symptomless. The pseudorecombinants 411 and 114 of isolates PV-0474 and PV-0184 caused mosaic symptoms while pseudorecombinant 141 was symptomless implying that RNA1 and RNA3 are responsible for symptomatology of isolate PV-0474.

The results demonstrate that symptom determinants differ for virus isolates and host species. No significant impact of RNA2 on symptomatology of the four investigated isolates was found in *N. benthamiana* and *C. sativus*, although RNA2 of CMV isolate Fny was described in literature as determining leaf distortion and stunting in *N. benthamiana* (Phan et al., 2014).

This work has established tests for CMV and tospoviruses based on the Luminex xTAG technology. They were compared with the standard procedures ELISA, RT-PCR and RFLP and found to be reliable for virus detection and distinction. After some adaptations the tests can be incorporated into broader assays for the simultaneous diagnosis of several plant pathogens expected in certain crops. This is an advantage the technique offers for routine diagnostics, but before adoption it will have to prove its reliability in high-throughput multiplex testing. This work additionally delivered four infectious full-length clones of CMV isolates from different subgroups with varying symptomatology, which can be helpful for further characterization of CMV. They can be used to study the determinants for symptomatology and also infectivity on different host plants. Functions of proteins and relevant protein domains can be specified through mutational studies with the full-length clones.

6 References

Adams, I.P., Glover, R.H., Monger, W.A., Mumford, R., Jackeviciene, E., Navalinskiene, M., Samuitiene, M., Boonham, N., 2009. Next-generation sequencing and metagenomic analysis: a universal diagnostic tool in plant virology. Molecular Plant Pathology 10 (4), 537–545.

Adkins, S., Quadt, R., Choi, T.J., Ahlquist, P., German, T., 1995. An RNA-dependent RNA polymerase activity associated with virions of tomato spotted wilt virus, a plant- and insect-infecting bunyavirus. Virology 207 (1), 308–311.

Ahlquist, P., Dasgupta, R., Kaesberg, P., 1981. Near identity of 3′ RNA secondary structure in bromoviruses and cucumber mosaic virus. Cell 23 (1), 183–189.

Avila, A.C. de, Haan, P. de, Kormelink, R., Resende, R.d.O., Goldbach, R.W., Peters, D., 1993. Classification of tospoviruses based on phylogeny of nucleoprotein gene sequences. The Journal of general virology 74 (Pt 2), 153–159.

Bag, S., Schwartz, H.F., Cramer, C.S., Havey, M.J., Pappu, H.R., 2015. Iris yellow spot virus (Tospovirus: Bunyaviridae): from obscurity to research priority. Molecular Plant Pathology 16 (3), 224–237.

Bentley, D.R., Balasubramanian, S., Swerdlow, H.P., Smith, G.P., Milton, J., Brown, C.G., Hall, K.P., Evers, D.J., Barnes, C.L., Bignell, H.R., Boutell, J.M., Bryant, J., Carter, R.J., Keira Cheetham, R., Cox, A.J., Ellis, D.J., Flatbush, M.R., Gormley, N.A., Humphray, S.J., Irving, L.J., Karbelashvili, M.S., Kirk, S.M., Li, H., Liu, X., Maisinger, K.S., Murray, L.J., Obradovic, B., Ost, T., Parkinson, M.L., Pratt, M.R., Rasolonjatovo, I.M.J., Reed, M.T., Rigatti, R., Rodighiero, C., Ross, M.T., Sabot, A., Sankar, S.V., Scally, A., Schroth, G.P., Smith, M.E., Smith, V.P., Spiridou, A., Torrance, P.E., Tzonev, S.S., Vermaas, E.H., Walter, K., Wu, X., Zhang, L., Alam, M.D., Anastasi, C., Aniebo, I.C., Bailey, D.M.D., Bancarz, I.R., Banerjee, S., Barbour, S.G., Baybayan, P.A., Benoit, V.A., Benson, K.F., Bevis, C., Black, P.J., Boodhun, A., Brennan, J.S., Bridgham, J.A., Brown, R.C., Brown, A.A., Buermann, D.H., Bundu, A.A., Burrows, J.C., Carter, N.P., Castillo, N., Chiara E Catenazzi, M., Chang, S., Neil Cooley, R., Crake, N.R., Dada, O.O., Diakoumakos, K.D., Dominguez-Fernandez, B., Earnshaw, D.J., Egbujor, U.C., Elmore, D.W., Etchin, S.S., Ewan, M.R., Fedurco, M., Fraser, L.J., Fuentes Fajardo, K.V., Scott Furey, W., George, D., Gietzen, K.J., Goddard, C.P., Golda, G.S., Granieri, P.A., Green, D.E., Gustafson, D.L., Hansen, N.F., Harnish, K., Haudenschild, C.D., Heyer, N.I., Hims, M.M., Ho, J.T., Horgan, A.M., Hoschler, K., Hurwitz, S., Ivanov, D.V., Johnson, M.Q., James, T., Huw Jones, T.A., Kang, G.-D., Kerelska, T.H., Kersey, A.D., Khrebtukova, I., Kindwall, A.P., Kingsbury, Z., Kokko-Gonzales, P.I., Kumar, A., Laurent, M.A., Lawley, C.T., Lee, S.E., Lee, X., Liao, A.K., Loch, J.A., Lok, M., Luo, S., Mammen, R.M., Martin, J.W., McCauley, P.G., McNitt, P., Mehta, P., Moon, K.W., Mullens, J.W., Newington, T., Ning, Z., Ling Ng, B., Novo, S.M., O'Neill, M.J., Osborne, M.A., Osnowski, A., Ostadan, O., Paraschos, L.L., Pickering, L., Pike, A.C., Pike, A.C., Chris Pinkard, D., Pliskin, D.P., Podhasky, J., Quijano, V.J., Raczy, C., Rae, V.H., Rawlings, S.R., Chiva Rodriguez, A., Roe, P.M., Rogers, J., Rogert Bacigalupo, M.C., Romanov, N., Romieu, A., Roth, R.K., Rourke, N.J., Ruediger, S.T., Rusman, E., Sanches-Kuiper, R.M., Schenker, M.R., Seoane, J.M., Shaw, R.J., Shiver, M.K., Short, S.W., Sizto, N.L., Sluis, J.P., Smith, M.A., Ernest Sohna Sohna, J., Spence, E.J., Stevens, K., Sutton, N., Szajkowski, L., Tregidgo, C.L., Turcatti, G., Vandevondele, S., Verhovsky, Y., Virk, S.M., Wakelin, S., Walcott, G.C., Wang, J., Worsley, G.J., Yan, J., Yau, L., Zuerlein, M., Rogers, J., Mullikin, J.C., Hurles, M.E., McCooke, N.J., West, J.S., Oaks, F.L., Lundberg, P.L., Klenerman, D., Durbin, R., Smith, A.J., 2008. Accurate whole human genome sequencing using reversible terminator chemistry. Nature 456 (7218), 53–59.

Bergervoet, J.H.W., Peters, J., van Beckhoven, J.R.C.M., van den Bovenkamp, G.W., Jacobson, J.W., van der Wolf, J.M., 2008. Multiplex microsphere immuno-detection of potato virus Y, X and PLRV. Journal of Virological Methods 149 (1), 63–68.

Bombarely, A., Rosli, H.G., Vrebalov, J., Moffett, P., Mueller, L.A., Martin, G.B., 2012. A draft genome sequence of Nicotiana benthamiana to enhance molecular plant-microbe biology research. Molecular plant-microbe interactions: MPMI 25 (12), 1523–1530.

Bonnet, J., Fraile, A., Sacristán, S., Malpica, J.M., García-Arenal, F., 2005. Role of recombination in the evolution of natural populations of Cucumber mosaic virus, a tripartite RNA plant virus. Virology 332 (1), 359–368.

Boonham, N., Kreuze, J., Winter, S., van der Vlugt, R., Bergervoet, J., Tomlinson, J., Mumford, R., 2014. Methods in virus diagnostics: from ELISA to next generation sequencing. Virus research 186, 20–31.

Boonham, N., Pérez, L., Mendez, M., Peralta, E., Blockley, A., Walsh, K., Barker, I., Mumford, R., 2004. Development of a real-time RT-PCR assay for the detection of Potato spindle tuber viroid. Journal of Virological Methods 116 (2), 139–146.

Boonham, N., Tomlinson, J., Mumford, R., 2007. Microarrays for rapid identification of plant viruses. Annual review of phytopathology 45, 307–328.

Botermans, M., van de Vossenberg, B. T. L. H., Verhoeven, J.T.J., Roenhorst, J.W., Hooftman, M., Dekter, R., Meekes, E.T.M., 2013. Development and validation of a real-time RT-PCR assay for generic detection of pospiviroids. Journal of Virological Methods 187 (1), 43–50.

Brunt, A.A., Crabtree, K., Dallwitz, M.J., Gibbs, A.J., Watson, L., Zurcher, E.J., 1997. Plant Viruses Online: Descriptions and Lists from the VIDE Database. http://sdb.im.ac.cn/vide. Accessed 4 May 2016.

Bujarski, J., Figlerowicz, M., Gallitelli, D., Roossinck, M.J. and Scott, S.W., 2012. Bromoviridae, in: King, A.M.Q., Adams, M.J., Carstens, E.B., Lefkowitz, E.J. (Ed.), Virus Taxonomy. Ninth Report of the International Committee on Taxonomy of Viruses. Elsevier, Amsterdam, pp. 965–976.

Byzova, N.A., Safenkova, I.V., Chirkov, S.N., Avdienko, V.G., Guseva, A.N., Mitrofanova, I.V., Zherdev, A.V., Dzantiev, B.B., Atabekov, J.G., 2010. Interaction of Plum Pox Virus with Specific Colloidal Gold-Labeled Antibodies and Development of Immunochromatographic Assay of the Virus. Biochemistry Moscow 75 (11), 1393–1403.

Carrère, I., Tepfer, M., Jacquemond, M., 1999. Recombinants of cucumber mosaic virus (CMV): determinants of host range and symptomatology. Archives of virology 144 (2), 365–379.

Charlermroj, R., Himananto, O., Seepiban, C., Kumpoosiri, M., Warin, N., Gajanandana, O., Elliott, C.T., Karoonuthaisiri, N., 2014. Antibody array in a multiwell plate format for the sensitive and multiplexed detection of important plant pathogens. Analytical chemistry 86 (14), 7049–7056.

Charoenvilaisiri, S., Seepiban, C., Bhunchoth, A., Warin, N., Luxananil, P., Gajanandana, O., 2014. Development of a multiplex RT-PCR-ELISA to identify four distinct species of tospovirus. Journal of Virological Methods 202, 54–63.

Chen, B., Francki, R.I.B., 1990. Cucumovirus transmission by the aphid Myzus persicae is determined solely by the viral coat protein. Journal of General Virology 71 (4), 939–944.

Chen, J., Tang, J., Liu, J., Cai, Z., Bai, X., 2012a. Development and evaluation of a multiplex PCR for simultaneous detection of five foodborne pathogens. Journal of applied microbiology 112 (4), 823–830.

Chen, K., Xu, Z., Yan, L., Wang, G., 2007a. Characterization of a New Strain of Capsicum chlorosis virus from Peanut (Arachis hypogaea L.) in China. J Phytopathol 155 (3), 178–181.

Chen, T.-C., Li, J.-T., Lin, Y.-P., Yeh, Y.-C., Kang, Y.-C., Huang, L.-H., Yeh, S.-D., 2012b. Genomic characterization of Calla lily chlorotic spot virus and design of broad-spectrum primers for detection of tospoviruses. Plant Pathology 61 (1), 183–194.

Chen, Y., Chen, J., Zhang, H., Tang, X., Du, Z., 2007b. Molecular evidence and sequence analysis of a natural reassortant between cucumber mosaic virus subgroup IA and II strains. Virus Genes 35 (2), 405–413.

Chu, F.-H., Chao, C.-H., Chung, M.-H., Chen, C.-C., Yeh, S.-D., 2001. Completion of the Genome Sequence of Watermelon silver mottle virus and Utilization of Degenerate Primers for Detecting Tospoviruses in Five Serogroups. Phytopathology 91 (4), 361–368.

Clark, M.F., Adams, A.N., 1977. Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. The Journal of general virology 34 (3), 475–483.

Compton, J., 1991. Nucleic acid sequence-based amplification. Nature 350 (6313), 91–92.

Croft, H., Malinowski, T., Krizbai, L., Mikec, I., Kajic, V., Reed, C., Varga, A., James, D., 2008. Use of Luminex xMAP-derived Bio-Plex bead-based suspension array for specific detection of PPV W and characterization of epitopes on the coat protein of the virus. Journal of Virological Methods 153 (2), 203–213.

Devergne, J. C., Cardin, L., 1975. Relations serologiques entre cucumovirus (CMV, TAV, PSV). Ann. Phytopathol. 7 (4), 255–276.

Deyong, Z., Willingmann, P., Heinze, C., Adam, G., Pfunder, M., Frey, B., Frey, J.E., 2005. Differentiation of Cucumber mosaic virus isolates by hybridization to oligonucleotides in a microarray format. Journal of Virological Methods 123 (1), 101–108.

Dietzgen, R.G., Sander, E., 1982. Monoclonal antibodies against a plant virus. Archives of virology 74 (2-3), 197–204.

Ding, B., Li, Q., Nguyen, L., Palukaitis, P., Lucas, W.J., 1995. Cucumber mosaic virus 3a protein potentiates cell-to-cell trafficking of CMV RNA in tobacco plants. Virology 207 (2), 345–353.

Ding, S.W., Anderson, B.J., Haase, H.R., Symons, R.H., 1994. New overlapping gene encoded by the cucumber mosaic virus genome. Virology 198 (2), 593–601.

Diveki, Z., Salanki, K., Balazs, E., 2004. The necrotic pathotype of the cucumber mosaic virus (CMV) ns strain is solely determined by amino acid 461 of the 1a protein. Molecular plant-microbe interactions: MPMI 17 (8), 837–845.

Drygin, Y.F., Blintsov, A.N., Grigorenko, V.G., Andreeva, I.P., Osipov, A.P., Varitzev, Y.A., Uskov, A.I., Kravchenko, D.V., Atabekov, J.G., 2012. Highly sensitive field test lateral flow immunodiagnostics of PVX infection. Applied microbiology and biotechnology 93 (1), 179–189.

Dufva, M., 2005. Fabrication of high quality microarrays. Biomolecular engineering 22 (5-6), 173–184.

Dunbar, S.A., Vander Zee, C.A., Oliver, K.G., Karem, K.L., Jacobson, J.W., 2003. Quantitative, multiplexed detection of bacterial pathogens: DNA and protein applications of the Luminex LabMAP system. Journal of microbiological methods 53 (2), 245–252.

Edwards, M.C., Fetch, T.G., Schwarz, P.B., Steffenson, B.J., 2001. Effect of Barley yellow dwarf virus Infection on Yield and Malting Quality of Barley. Plant Disease 85 (2), 202–207.

Edwards, M. C., Gonsalves, D., 1983. Grouping of seven biologically defined isolates of cucumber mosaic virus by peptide mapping. Phytopathology 73 (11), 17.

Eid, J., Fehr, A., Gray, J., Luong, K., Lyle, J., Otto, G., Peluso, P., Rank, D., Baybayan, P., Bettman, B., Bibillo, A., Bjornson, K., Chaudhuri, B., Christians, F., Cicero, R., Clark, S., Dalal, R., Dewinter, A., Dixon, J., Foquet, M., Gaertner, A., Hardenbol, P., Heiner, C., Hester, K., Holden, D., Kearns, G., Kong, X., Kuse, R., Lacroix, Y., Lin, S., Lundquist, P., Ma, C., Marks, P., Maxham, M., Murphy, D., Park, I., Pham, T., Phillips, M., Roy, J., Sebra, R., Shen, G., Sorenson, J., Tomaney, A., Travers, K., Trulson, M., Vieceli, J., Wegener, J., Wu, D., Yang, A., Zaccarin, D., Zhao, P., Zhong, F., Korlach, J., Turner, S., 2009. Real-time DNA sequencing from single polymerase molecules. Science (New York, N.Y.) 323 (5910), 133–138.

Eigen, M., 1993. Viral quasispecies. Scientific American 269 (1), 42–49.

Essafi, A., Diaz-Pendon, J.A., Moriones, E., Monforte, A.J., Garcia-Mas, J., Martin-Hernandez, A.M., 2009. Dissection of the oligogenic resistance to Cucumber mosaic virus in the melon accession PI 161375. TAG. Theoretical and applied genetics. Theoretische und angewandte Genetik 118 (2), 275–284.

European and Mediterranean Plant Protection Organization (EPPO), 2015. EPPO A1 and A2 lists of pests recommended for regulation as quarantine pests. http://archives.eppo.int/EPPOStandards/PM1_GENERAL/pm1-02(24)_A1A2_2015.pdf. Accessed 9 August 2016.

Felsenstein, J., 1985. Confidence Limits on Phylogenies: An Approach Using the Bootstrap. Evolution 39 (4), 783.

Finch, J.T., Klug, A., van Regenmortel, M H, 1967. The structure of cucumber mosaic virus. Journal of molecular biology 24 (2), 303–305.

Fukuta, S., Iida, T., Mizukami, Y., Ishida, A., Ueda, J., Kanbe, M., Ishimoto, Y., 2003. Detection of Japanese yam mosaic virus by RT-LAMP. Archives of virology 148 (9), 1713–1720.

Gahoi, S., Arya, L., Anil, R., Marla, S., 2013. DPPrimer - A Degenerate PCR Primer Design Tool. Bioinformation 9 (18), 937–940.

Gallitelli, D., 2000. The ecology of Cucumber mosaic virus and sustainable agriculture. Virus research 71 (1-2), 9–21.

Gibson, D.G., Young, L., Chuang, R.-Y., Venter, J.C., Hutchison, C.A., Smith, H.O., 2009. Enzymatic assembly of DNA molecules up to several hundred kilobases. Nat Meth 6 (5), 343–345.

Gould, A.R., Symons, R.H., 1982. Cucumber Mosaic Virus RNA 3. European Journal of Biochemistry 126 (2), 217–226.

Groves, C., German, T., Dasgupta, R., Mueller, D., Smith, D.L., 2016. Seed Transmission of Soybean vein necrosis virus: The First Tospovirus Implicated in Seed Transmission. PLoS ONE 11 (1), e0147342.

Guo, H.S., Ding, S.W., 2002. A viral protein inhibits the long range signaling activity of the gene silencing signal. The EMBO journal 21 (3), 398–407.

Habili, N., Symons, R.H., 1989. Evolutionary relationship between luteoviruses and other RNA plant viruses based on sequence motifs in their putative RNA polymerases and nucleic acid helicases. Nucl Acids Res 17 (23), 9543–9555.

Hadersdorfer, J., Neumüller, M., Treutter, D., Fischer, T.C., 2011. Fast and reliable detection of Plum pox virus in woody host plants using the Blue LAMP protocol. Annals of Applied Biology 159 (3), 456–466.

Hagen, C., Frizzi, A., Gabriels, S., Huang, M., Salati, R., Gabor, B., Huang, S., 2012. Accurate and sensitive diagnosis of geminiviruses through enrichment, high-throughput sequencing and automated sequence identification. Archives of virology 157 (5), 907–915.

Haible, D., Kober, S., Jeske, H., 2006. Rolling circle amplification revolutionizes diagnosis and genomics of geminiviruses. Journal of Virological Methods 135 (1), 9–16.

Hanahan, D., 1983. Studies on transformation of Escherichia coli with plasmids. Journal of molecular biology 166 (4), 557–580.

Hanssen, I.M., Lapidot, M., Thomma, B.P., 2010. Emerging viral diseases of tomato crops. Molecular plant-microbe interactions : MPMI 23 (5), 539–548.

Hardy, B., 1996. Major potato diseases, insects, and nematodes. International Potato Center, Lima.

Hassani-Mehraban, A., Botermans, M., Verhoeven, J.T.J., Meekes, E., Saaijer, J., Peters, D., Goldbach, R., Kormelink, R., 2010. A distinct tospovirus causing necrotic streak on Alstroemeria sp. in Colombia. Archives of virology 155 (3), 423–428.

Hassani-Mehraban, A., Westenberg, M., Verhoeven, J.T.J., van de Vossenberg B.T.L.H., Kormelink R., Roenhorst J. W., 2016. Generic RT-PCR tests for detection and identification of tospoviruses. Journal of Virological Methods 233, 89–96.

Hayes, R.J., Buck, K.W., 1990. Complete replication of a eukaryotic virus RNA in vitro by a purified RNA-dependent RNA polymerase. Cell 63 (2), 363–368.

Heid, C.A., Stevens, J., Livak, K.J., Williams, P.M., 1996. Real time quantitative PCR. Genome Research 6 (10), 986–994.

Hsu, H.T., Barzuna, L., Hsu, Y.H., Bliss, W., Perry, K.L., 2000. Identification and Subgrouping of Cucumber mosaic virus with Mouse Monoclonal Antibodies. Phytopathology 90 (6), 615–620.

Hu, Q., Lyu, D., Shi, X., Jiang, Y., Lin, Y., Li, Y., Qiu, Y., He, L., Zhang, R., Li, Q., 2014. A modified molecular beacons-based multiplex real-time PCR assay for simultaneous detection of eight foodborne pathogens in a single reaction and its application. Foodborne pathogens and disease 11 (3), 207–214.

Huang, A., Qiu, Z., Jin, M., Shen, Z., Chen, Z., Wang, X., Li, J.-W., 2014. High-throughput detection of food-borne pathogenic bacteria using oligonucleotide microarray with quantum dots as fluorescent labels. International journal of food microbiology 185, 27–32.

Huang, Q., Baum, L., Fu, W.L., 2010. Simple and practical staining of DNA with GelRed in agarose gel electrophoresis. Clinical laboratory 56 (3-4), 149–152.

Huang, S., Li, R., Zhang, Z., Li, L., Gu, X., Fan, W., Lucas, W.J., Wang, X., Xie, B., Ni, P., Ren, Y., Zhu, H., Li, J., Lin, K., Jin, W., Fei, Z., Li, G., Staub, J., Kilian, A., van der Vossen, Edwin A G, Wu, Y., Guo, J., He, J., Jia, Z., Ren, Y., Tian, G., Lu, Y., Ruan, J., Qian, W., Wang, M., Huang, Q., Li, B., Xuan, Z., Cao, J., Asan, Wu, Z., Zhang, J., Cai, Q., Bai, Y., Zhao, B., Han, Y., Li, Y., Li, X., Wang, S., Shi, Q., Liu, S., Cho, W.K., Kim, J.-Y., Xu, Y., Heller-Uszynska, K., Miao, H., Cheng, Z., Zhang, S., Wu, J., Yang, Y., Kang, H., Li, M., Liang, H., Ren, X., Shi, Z., Wen, M., Jian, M., Yang, H., Zhang, G., Yang, Z., Chen, R., Liu, S., Li, J., Ma, L., Liu, H., Zhou, Y., Zhao, J., Fang, X., Li, G., Fang, L., Li, Y., Liu, D., Zheng, H., Zhang, Y., Qin, N., Li, Z., Yang, G., Yang, S., Bolund, L., Kristiansen, K., Zheng, H., Li, S., Zhang, X., Yang, H., Wang, J., Sun, R., Zhang, B., Jiang, S., Wang, J., Du, Y., Li, S., 2009. The genome of the cucumber, *Cucumis sativus* L. Nature genetics 41 (12), 1275–1281.

Hussa, R.O., Hudson, E.N., 1984. A two-site immunometric assay in evaluation of low levels of serum hCG. Am Clin Prod Rev 3 (12), 12–17.

International Committee on Taxonomy of Viruses, 2015. Master Species List #30. http://www.ictvonline.org/virusTaxonomy.asp. Accessed 14 July 2016.

Jacquemond, M., 2012. Cucumber mosaic virus. Advances in virus research 84, 439–504.

Jin, Y.O., Mattes, T.E., 2011. Assessment and modification of degenerate qPCR primers that amplify functional genes from etheneotrophs and vinyl chloride-assimilators. Letters in applied microbiology 53 (5), 576–580.

Kaneko, H., Kawana, T., Fukushima, E., Suzutani, T., 2007. Tolerance of loop-mediated isothermal amplification to a culture medium and biological substances. Journal of biochemical and biophysical methods 70 (3), 499–501.

Kaper, J., Tousignant, M., 1984. Viral satellites: Parasitic nucleic acids capable of modulating disease expression. Endeavour 8 (4), 194–200.

Kirk, W.D.J., Terry, L.I., 2003. The spread of the western flower thrips Frankliniella occidentalis (Pergande). Agric Forest Ent 5 (4), 301–310.

Köhler, G., Milstein, C., 1975. Continuous cultures of fused cells secreting antibody of predefined specificity. Nature 256 (5517), 495–497.

Kormelink, R., Garcia, M.L., Goodin, M., Sasaya, T., Haenni, A.-L., 2011. Negative-strand RNA viruses: the plant-infecting counterparts. Virus research 162 (1-2), 184–202.

Kormelink, R., Haan, P. de, Meurs, C., Peters, D., Goldbach, R., 1992a. The nucleotide sequence of the M RNA segment of tomato spotted wilt virus, a bunyavirus with two ambisense RNA segments. The Journal of general virology 73 (Pt 11), 2795–2804.

Kormelink, R., van Poelwijk, F., Peters, D., Goldbach, R., 1992b. Non-viral heterogeneous sequences at the 5' ends of tomato spotted wilt virus mRNAs. The Journal of general virology 73 (Pt 8), 2125–2128.

Kreuze, J.F., Perez, A., Untiveros, M., Quispe, D., Fuentes, S., Barker, I., Simon, R., 2009. Complete viral genome sequence and discovery of novel viruses by deep sequencing of small RNAs: a generic method for diagnosis, discovery and sequencing of viruses. Virology 388 (1), 1–7.

Kumar, S., Gautam, K.K., RAJ, S.K., 2015. Sequence analysis and genetic diversity of five new Indian isolates of cucumber mosaic virus. av 59 (04), 398–404.

Kuwabara, K., Yokoi, N., Ohki, T., Tsuda, S., 2010. Improved multiplex reverse transcription-polymerase chain reaction to detect and identify five tospovirus species simultaneously. J Gen Plant Pathol 76 (4), 273–277.

Law, M.D., Moyer, J.W., 1990. A tomato spotted wilt-like virus with a serologically distinct N protein. Journal of General Virology 71 (4), 933–938.

Legg, J., 1999. Emergence, spread and strategies for controlling the pandemic of cassava mosaic virus disease in east and central Africa. Crop Protection 18 (10), 627–637.

Leone, G., van Schijndel, H.B., van Genien, B., Schoen, C.D., 1997. Direct detection of potato leafroll virus in potato tubers by immunocapture and the isothermal nucleic acid amplification method NASBA. Journal of Virological Methods 66 (1), 19–27.

Lewandowski, D.J., Adkins, S., 2005. The tubule-forming NSm protein from Tomato spotted wilt virus complements cell-to-cell and long-distance movement of Tobacco mosaic virus hybrids. Virology 342 (1), 26–37.

Lewsey, M., Robertson, F.C., Canto, T., Palukaitis, P., Carr, J.P., 2007. Selective targeting of miRNA-regulated plant development by a viral counter-silencing protein. The Plant journal : for cell and molecular biology 50 (2), 240–252.

Lie, Y.S., Petropoulos, C.J., 1998. Advances in quantitative PCR technology: 5′ nuclease assays. Current Opinion in Biotechnology 9 (1), 43–48.

Lim, M.S., Kim, S.M., Choi, S.H., 2016. Simultaneous detection of three lily-infecting viruses using a multiplex Luminex bead array. Journal of Virological Methods 231, 34–37.

Lin, A., Nguyen, L., Lee, T., Clotilde, L.M., Kase, J.A., Son, I., Carter, J.M., Lauzon, C.R., 2011. Rapid O serogroup identification of the ten most clinically relevant STECs by Luminex microbead-based suspension array. Journal of microbiological methods 87 (1), 105–110.

Lin, H.-X., Rubio, L., Smythe, A., Jiminez, M., Falk, B.W., 2003. Genetic diversity and biological variation among California isolates of Cucumber mosaic virus. The Journal of general virology 84 (Pt 1), 249–258.

Linhart, C., Shamir, R., 2007. Degenerate primer design: theoretical analysis and the HYDEN program. Methods in molecular biology (Clifton, N.J.) 402, 221–244.

Liu, J., Kibiki, G., Maro, V., Maro, A., Kumburu, H., Swai, N., Taniuchi, M., Gratz, J., Toney, D., Kang, G., Houpt, E., 2011. Multiplex reverse transcription PCR Luminex assay for detection and quantitation of viral agents of gastroenteritis. Journal of clinical virology : the official publication of the Pan American Society for Clinical Virology 50 (4), 308–313.

Lot, H., Kaper, J.M., 1976. Further studies on the RNA component distribution among the nucleoproteins of cucumber mosaic virus. Virology 74 (1), 223–226.

Mahony, J., Chong, S., Merante, F., Yaghoubian, S., Sinha, T., Lisle, C., Janeczko, R., 2007. Development of a Respiratory Virus Panel Test for Detection of Twenty Human Respiratory Viruses by Use of Multiplex PCR and a Fluid Microbead-Based Assay. Journal of Clinical Microbiology 45 (9), 2965–2970.

Mandal, B., Jain, R.K., Krishnareddy, M., Krishna Kumar, N.K., Ravi, K.S., Pappu, H.R., 2012. Emerging Problems of Tospoviruses (Bunyaviridae) and their Management in the Indian Subcontinent. Plant Disease 96 (4), 468–479.

Margulies, M., Egholm, M., Altman, W.E., Attiya, S., Bader, J.S., Bemben, L.A., Berka, J., Braverman, M.S., Chen, Y.-J., Chen, Z., Dewell, S.B., Du, L., Fierro, J.M., Gomes, X.V., Godwin, B.C., He, W., Helgesen, S., Ho, C.H., Irzyk, G.P., Jando, S.C., Alenquer, M.L.I., Jarvie, T.P., Jirage, K.B., Kim, J.-B., Knight, J.R., Lanza, J.R., Leamon, J.H., Lefkowitz, S.M., Lei, M., Li, J., Lohman, K.L., Lu, H., Makhijani, V.B., McDade, K.E., McKenna, M.P., Myers, E.W., Nickerson, E., Nobile, J.R., Plant, R., Puc, B.P., Ronan, M.T., Roth, G.T., Sarkis, G.J., Simons, J.F., Simpson, J.W., Srinivasan, M., Tartaro, K.R., Tomasz, A., Vogt, K.A., Volkmer, G.A., Wang, S.H., Wang, Y., Weiner, M.P., Yu, P., Begley, R.F., Rothberg, J.M., 2005. Genome sequencing in microfabricated high-density picolitre reactors. Nature 437 (7057), 376–380.

Mattanovich, D., Ruker, F., Machado, A.C., Laimer, M., Regner, F., Steinkellner, H., Himmler, G., Katinger, H., 1989. Efficient transformation of Agrobacterium spp. by electroporation. Nucleic Acids Research 17 (16), 6747.

McMichael, L.A., Persley, D.M., Thomas, J.E., 2002. A new tospovirus serogroup IV species infecting capsicum and tomato in Queensland, Australia. Austral. Plant Pathol. 31 (3), 231–239.

Menzel, W., Jelkmann, W., Maiss, E., 2002. Detection of four apple viruses by multiplex RT-PCR assays with coamplification of plant mRNA as internal control. Journal of Virological Methods 99 (1-2), 81–92.

Mochizuki, T., Ohki, S.T., 2012. Cucumber mosaic virus: viral genes as virulence determinants. Molecular Plant Pathology 13 (3), 217–225.

Mumford, R.A., Walsh, K., Barker, I., Boonham, N., 2000. Detection of Potato mop top virus and Tobacco rattle virus Using a Multiplex Real-Time Fluorescent Reverse-Transcription Polymerase Chain Reaction Assay. Phytopathology 90 (5), 448–453.

Murai, T., 2001. Life history study of Thrips setosus. Entomologia Experimentalis et Applicata 100 (2), 245–251.

Nagamine, K., Watanabe, K., Ohtsuka, K., Hase, T., Notomi, T., 2001. Loop-mediated isothermal amplification reaction using a nondenatured template. Clinical chemistry 47 (9), 1742–1743.

Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N., Hase, T., 2000. Loop-mediated isothermal amplification of DNA. Nucleic Acids Research 28 (12), E63.

Nouri, S., Arevalo, R., Falk, B.W., Groves, R.L., Martin, D.P., 2014. Genetic Structure and Molecular Variability of Cucumber mosaic virus Isolates in the United States. PLoS ONE 9 (5), e96582.

Osman, F., Hodzic, E., Kwon, S.-J., Wang, J., Vidalakis, G., 2015. Development and validation of a multiplex reverse transcription quantitative PCR (RT-qPCR) assay for the rapid detection of Citrus tristeza virus, Citrus psorosis virus, and Citrus leaf blotch virus. Journal of Virological Methods 220, 64–75.

Owen, J., Palukaitis, P., 1988. Characterization of cucumber mosaic virus I. Molecular heterogeneity mapping of RNA 3 in eight CMV strains. Virology 166 (2), 495–502.

Palukaitis, P., Roossinck, M.J., Dietzgen, R.G., Francki, R.I., 1992. Cucumber mosaic virus, in: Maramorosch, K., Murphy, F.A., Shatkin, A.J. (Eds.), Advances in Virus Research, vol. 41. Academic Press, San Diego, pp. 281–348.

Pappu, H.R., Jones, R.A.C., Jain, R.K., 2009. Global status of tospovirus epidemics in diverse cropping systems: successes achieved and challenges ahead. Virus research 141 (2), 219–236.

Paret, M.L., Vallad, G.E., Zhang, S., Jones, J.B., 2015. Proceedings of the IVth international symposium on tomato diseases: Orlando, Florida, USA, June 24-27, 2013. ISHS, Leuven, Belgium, 348 pp.

Parrella, G., Gognalons, P., Gebre-Selassiè, K., Vovlas, C., Marchoux, G., 2003. An update of the host range of Tomato spotted wilt virus. Journal of Plant Pathology 85 (4), 227–264.

Peden, K., Symons, R.H., 1973. Cucumber mosaic virus contains a functionally divided genome. Virology 53 (2), 487–492.

Phan, M.S.V., Seo, J.-K., Choi, H.-S., Lee, S.-H., Kim, K.-H., 2014. Pseudorecombination between Two Distinct Strains of Cucumber mosaic virus Results in Enhancement of Symptom Severity. The Plant Pathology Journal 30 (3), 316–322.

Piazzolla, P., Crescenzi, A., Biasi, M. de, Tamburro, A.M., 1998. Circular dichroism studies of CMV-D and CMV-S: Two strains of cucumber mosaic cucumovirus with a different biological behaviour. Arch. Virol. 143 (12), 2305–2312.

Piepenburg, O., Williams, C.H., Stemple, D.L., Armes, N.A., 2006. DNA detection using recombination proteins. PLoS biology 4 (7), e204.

Plyusnin, A., Beaty, B.J., Elliott, R.M., Goldbach, R., Kormelink, R., Lundkvist, A., Schmaljohn, C.S., Tesh, R.B., 2012. Bunyaviridae, in: King, A.M.Q., Adams, M.J., Carstens, E.B., Lefkowitz, E.J. (Ed.), Virus taxonomy. Ninth report of the International Committee on Taxonomy of Viruses. Elsevier Academic Press, London, pp. 725–741.

Quesada-Gonzalez, D., Merkoci, A., 2015. Nanoparticle-based lateral flow biosensors. Biosensors & bioelectronics 73, 47–63.

Rao, A.L.N., Francki, R.I.B., 1982. Distribution of Determinants for Symptom Production and Host Range on the Three RNA Components of Cucumber Mosaic Virus. Journal of General Virology 61 (2), 197–205.

Rao, R.S., Visuri, S.R., McBride, M.T., Albala, J.S., Matthews, D.L., Coleman, M.A., 2004. Comparison of Multiplexed Techniques for Detection of Bacterial and Viral Proteins. J. Proteome Res. 3 (4), 736–742.

Rasoulpour, R., Afsharifar, A., Izadpanah, K., 2016. Partial biological and molecular characterization of a Cucumber mosaic virus isolate naturally infecting Cucumis melo in Iran. Virusdisease 27 (2), 193–197.

Rezaian, M.A., Williams, R.H.V., Gordon, K.H.J., Gould, A.R., Symons, R.H., 1984. Nucleotide sequence of cucumber-mosaic-virus RNA 2 reveals a translation product significantly homologous to corresponding proteins of other viruses. Eur J Biochem 143 (2), 277–284.

Rezaian, M.A., Williams, R.H.V., Symons, R.H., 1985. Nucleotide sequence of cucumber mosaic virus RNA 1. Presence of a sequence complementary to part of the viral satellite RNA and homologies with other viral RNAs. Eur J Biochem 150 (2), 331–339.

Roossinck, M.J., 2001. Cucumber mosaic virus, a model for RNA virus evolution. Molecular Plant Pathology 2 (2), 59–63.

Roossinck, M.J., 2002. Evolutionary History of Cucumber Mosaic Virus Deduced by Phylogenetic Analyses. Journal of virology 76 (7), 3382–3387.

Roossinck, M.J., Zhang, L., Hellwald, K.H., 1999. Rearrangements in the 5' nontranslated region and phylogenetic analyses of cucumber mosaic virus RNA 3 indicate radial evolution of three subgroups. Journal of virology 73 (8), 6752–6758.

Rotenberg, D., Jacobson, A.L., Schneweis, D.J., Whitfield, A.E., 2015. Thrips transmission of tospoviruses. Current Opinion in Virology 15, 80–89.

Rothberg, J.M., Hinz, W., Rearick, T.M., Schultz, J., Mileski, W., Davey, M., Leamon, J.H., Johnson, K., Milgrew, M.J., Edwards, M., Hoon, J., Simons, J.F., Marran, D., Myers, J.W., Davidson, J.F., Branting, A., Nobile, J.R., Puc, B.P., Light, D., Clark, T.A., Huber, M., Branciforte, J.T., Stoner, I.B., Cawley, S.E., Lyons, M., Fu, Y., Homer, N., Sedova, M., Miao, X., Reed, B., Sabina, J., Feierstein, E., Schorn, M., Alanjary, M., Dimalanta, E., Dressman, D., Kasinskas, R., Sokolsky, T., Fidanza, J.A., Namsaraev, E., McKernan, K.J., Williams, A., Roth, G.T., Bustillo, J., 2011. An integrated semiconductor device enabling non-optical genome sequencing. Nature 475 (7356), 348–352.

Rozanov, M.N., Koonin, E.V., Gorbalenya, A.E., 1992. Conservation of the putative methyltransferase domain: a hallmark of the 'Sindbis-like' supergroup of positive-strand RNA viruses. The Journal of general virology 73 (Pt 8), 2129–2134.

Rybicki, E.P., 2015. A Top Ten list for economically important plant viruses. Archives of virology 160 (1), 17–20.

Ryu, J., Park, S.H., Yeom, Y.S., Shrivastav, A., Lee, S.-H., Kim, Y.-R., Kim, H.-Y., 2013. Simultaneous detection of Listeria species isolated from meat processed foods using multiplex PCR. Food Control 32 (2), 659–664.

Saiki, R., Gelfand, D., Stoffel, S., Scharf, S., Higuchi, R., Horn, G., Mullis, K., Erlich, H., 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 239 (4839), 487–491.

Saitou, N., Nei, M., 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Molecular biology and evolution 4 (4), 406–425.

Samuel, G., Bald, J.G., Pittman, H.A., 1930. Investigations on 'spotted wilt' of Tomatoes in Australia. Australian Council for Scientific and Industrial Research Bulletin 44, 8–11.

Schena, M., Shalon, D., Davis, R.W., Brown, P.O., 1995. Quantitative monitoring of gene expression patterns with a complementary DNA microarray. Science (New York, N.Y.) 270 (5235), 467–470.

Schmelzer, K., 1957. Beiträge zur Kenntnis der Übertragbarkeit von Viren durch Cuscuta-Arten. Phytopathologische Zeitschrift (28), 1–56.

Scholthof, K.-B.G., Adkins, S., Czosnek, H., Palukaitis, P., Jacquot, E., Hohn, T., Hohn, B., Saunders, K., Candresse, T., Ahlquist, P., Hemenway, C., Foster, G.D., 2011. Top 10 plant viruses in molecular plant pathology. Molecular Plant Pathology 12 (9), 938–954.

Schwinghamer, M.W., Symons, R.H., 1977. Translation of the four major RNA species of cucumber mosaic virus in plant and animal cell-free systems and in toad oocytes. Virology 79 (1), 88–108.

Severgnini, M., Cremonesi, P., Consolandi, C., Bellis, G. de, Castiglioni, B., 2011. Advances in DNA Microarray Technology for the Detection of Foodborne Pathogens. Food Bioprocess Technol 4 (6), 936–953.

Shendure, J., Porreca, G.J., Reppas, N.B., Lin, X., McCutcheon, J.P., Rosenbaum, A.M., Wang, M.D., Zhang, K., Mitra, R.D., Church, G.M., 2005. Accurate multiplex polony sequencing of an evolved bacterial genome. Science (New York, N.Y.) 309 (5741), 1728–1732.

Shintaku, M., 1991. Coat protein gene sequences of two cucumber mosaic virus strains reveal a single amino acid change correlating with chlorosis induction. The Journal of general virology 72 (Pt 10), 2587–2589.

Shintaku, M., Palukaitis, P., 1990. Genetic Mapping of Cucumber Mosaic Virus, in: Leigh, J. (Ed.), Viral genes and plant pathogenesis. Springer, [Place of publication not identified], pp. 156–164.

Shintaku, M.H., Zhang, L., Palukaitis, P., 1992. A single amino acid substitution in the coat protein of cucumber mosaic virus induces chlorosis in tobacco. The Plant cell 4 (7), 751–757.

Sleat, D.E., Zhang, L., Palukaitis, P., 1994. Mapping determinants within cucumber mosaic virus and its satellite RNA for the induction of necrosis in tomato plants. Molecular plant-microbe interactions : MPMI 7 (2), 189–195.

Song, S., Liu, N., Zhao, Z., Njumbe Ediage, E., Wu, S., Sun, C., Saeger, S. de, Wu, A., 2014. Multiplex lateral flow immunoassay for mycotoxin determination. Analytical chemistry 86 (10), 4995–5001.

Staheli, J.P., Boyce, R., Kovarik, D., Rose, T.M., 2011. CODEHOP PCR and CODEHOP PCR primer design. Methods in molecular biology (Clifton, N.J.) 687, 57–73.

Stephan, D., 2005. Molekulare Charakterisierung von Beet mild yellowing virus (BMYV) und Beet chlorosis virus (BChV) sowie Selektion von BMYV Amplicon-transgenen *Nicotiana benthamiana*. Dissertation, Hannover.

Suzuki, M., Kuwata, S., Kataoka, J., Masuta, C., Nitta, N., Takanami, Y., 1991. Functional analysis of deletion mutants of cucumber mosaic virus RNA3 using an in vitro transcription system. Virology 183 (1), 106–113.

Symons, R.H., 1975. Cucumber mosaic virus RNA contains 7-methyl guanosine at the 5'-terminus of all four RNA species. Molecular biology reports 2 (4), 277–285.

Takeda, A., Sugiyama, K., Nagano, H., Mori, M., Kaido, M., Mise, K., Tsuda, S., Okuno, T., 2002. Identification of a novel RNA silencing suppressor, NSs protein of Tomato spotted wilt virus. FEBS Letters 532 (1-2), 75–79.

Tamura, K., Nei, M., Kumar, S., 2004. Prospects for inferring very large phylogenies by using the neighbor-joining method. Proceedings of the National Academy of Sciences of the United States of America 101 (30), 11030–11035.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S., 2013. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Molecular biology and evolution 30 (12), 2725–2729.

Tentchev, D., Verdin, E., Marchal, C., Jacquet, M., Aguilar, J.M., Moury, B., 2011. Evolution and structure of Tomato spotted wilt virus populations: evidence of extensive reassortment and insights into emergence processes. The Journal of general virology 92 (Pt 4), 961–973.

The Tomato Genome Consortium, 2012. The tomato genome sequence provides insights into fleshy fruit evolution. Nature 485 (7400), 635–641.

Torrance, L., Jones, R.A.C., 1981. Recent developments in serological methods suited for use in routine testing for plant viruses. Plant Pathology 30 (1), 1–24.

Tripathi, D., Raikhy, G., Pappu, H.R., 2015. Movement and nucleocapsid proteins coded by two tospovirus species interact through multiple binding regions in mixed infections. Virology 478, 137–147.

Tsuda, S., Kameya-Iwaki, M., Hanada, K., Kouda, Y., Hikata, M., Tomaru, K., 1992. A novel detection and identification technique for plant viruses: rapid immunofilter paper assay (RIPA). Plant Disease 76, 466–469.

Turina, M., Tavella, L., Ciuffo, M., 2012. Tospoviruses in the Mediterranean Area, in: Loebenstein, G., Lecoq, H. (Ed.), Advances in Virus Research. Viruses and Virus Diseases of Vegetables in the Mediterranean Basin, vol. 84. Elsevier Academic Press, San Diego, California, pp. 403–437.

Uga, H., Tsuda, S., 2005. A One-Step Reverse Transcription-Polymerase Chain Reaction System for the Simultaneous Detection and Identification of Multiple Tospovirus Infections. Phytopathology 95 (2), 166–171.

United Nations Economic Commission for Europe (UNECE), 2014. UNECE Standard S-1 concerning the marketing and commercial quality control of seed potatoes. http://www.unece.org/fileadmin/DAM/trade/agr/standard/potatoes/S-1_SeedPotatoes_2014_e.pdf. Accessed 4 August 2016.

Usuda, S., Okamoto, H., Iwanari, H., Baba, K., Tsuda, F., Miyakawa, Y., Mayumi, M., 1999. Serological detection of hepatitis B virus genotypes by ELISA with monoclonal antibodies to type-specific epitopes in the preS2-region product. Journal of Virological Methods 80 (1), 97–112.

van Brunschot, S.L., Bergervoet, J.H.W., Pagendam, D.E., Weerdt, M. de, Geering, A.D.W., Drenth, A., van der Vlugt, R.A.A., 2014a. A bead-based suspension array for the multiplexed detection of begomoviruses and their whitefly vectors. Journal of Virological Methods 198, 86–94.

van Brunschot, S.L., Bergervoet J.H.W., Pagendam D.E., Weerdt M. de, Geering A.D.W., Drenth A., van der Vlugt R.A.A., 2014b. Development of a multiplexed bead-based suspension array for the detection and discrimination of pospiviroid plant pathogens. PLoS ONE 9 (1), e84743.

van den Heuvel, J.F.J.M., van der Vlugt, R.A.A., Verbeek, M., Haan, P.T. de, Huttinga, H., 1994. Characteristics of a resistance-breaking isolate of potato virus Y causing potato tuber necrotic ringspot disease. Eur J Plant Pathol 100 (5), 347–356.

van der Vlugt, R.A.A., van Raaij, H., Weerdt, M. de, Bergervoet, J.H.W., 2015. Multiplex detection of plant pathogens through the Luminex MagPlex bead system. Methods in molecular biology (Clifton, N.J.) 1302, 283–299.

van Regenmortel, M.H., Hendry, D.A., Baltz, T., 1972. A reexamination of the molecular size of cucumber mosaic virus and its coat protein. Virology 49 (3), 647–653.

Vincent, M., Xu, Y., Kong, H., 2004. Helicase-dependent isothermal DNA amplification. EMBO reports 5 (8), 795–800.

Voinnet, O., Rivas, S., Mestre, P., Baulcombe, D., 2003. Retracted: An enhanced transient expression system in plants based on suppression of gene silencing by the p19 protein of tomato bushy stunt virus. The Plant Journal 33 (5), 949–956.

Voller, A., Bartlett, A., Bidwell, D.E., Clark, M.F., Adams, A.N., 1976. The detection of viruses by enzyme-linked immunosorbent assay (ELISA). The Journal of general virology 33 (1), 165–167.

Voller, A., Bidwell, D., Huldt, G., Engvall, E., 1974. A microplate method of enzyme-linked immunosorbent assay and its application to malaria. Bulletin of the World Health Organization 51 (2), 209–211.

Vunsh, R.O.N., Rosner, A., Stein, A., 1990. The use of the polymerase chain reaction (PCR) for the detection of bean yellow mosaic virus in gladiolus. Ann Applied Biology 117 (3), 561–569.

Wang, Y., Gaba, V., Yang, J., Palukaitis, P., Gal-On, A., 2002. Characterization of Synergy Between Cucumber mosaic virus and Potyviruses in Cucurbit Hosts. Phytopathology 92 (1), 51–58.

Wastling, S.L., Picozzi, K., Kakembo, A.S.L., Welburn, S.C., 2010. LAMP for human African trypanosomiasis: a comparative study of detection formats. PLoS neglected tropical diseases 4 (11), e865.

Webster, C.G., Frantz, G., Reitz, S.R., Funderburk, J.E., Mellinger, H.C., McAvoy, E., Turechek, W.W., Marshall, S.H., Tantiwanich, Y., McGrath, M.T., Daughtrey, M.L., Adkins, S., 2015. Emergence of Groundnut ringspot virus and Tomato chlorotic spot virus in Vegetables in Florida and the Southeastern United States. Phytopathology 105 (3), 388–398.

Webster, C.G., Reitz, S.R., Perry, K.L., Adkins, S., 2011. A natural M RNA reassortant arising from two species of plant- and insect-infecting bunyaviruses and comparison of its sequence and biological properties to parental species. Virology 413 (2), 216–225.

Weidemann, H. L., and Maiss E., 1996. Detection of the Potato Tuber Necrotic Ringspot Strain of Potato Virus Y (PVY NTN) by Reverse Transcription and Immunocapture Polymerase Chain Reaction. Journal of Plant Diseases and Protection 103 (4), 337–345.

White, P., Morales, F., Roossinck, M.J., 1995. Interspecific Reassortment of Genomic Segments in the Evolution of Cucumoviruses. Virology 207 (1), 334–337.

Wylie, S.J., Jones, M.G.K., 2011. The complete genome sequence of a Passion fruit woodiness virus isolate from Australia determined using deep sequencing, and its relationship to other potyviruses. Archives of virology 156 (3), 479–482.

Xiang, C., Han, P., Lutziger, I., Wang, K., Oliver, D.J., 1999. A mini binary vector series for plant transformation. Plant molecular biology 40 (4), 711–717.

Yang, Y., Kim, K.S., Anderson, E.J., 1997. Seed Transmission of Cucumber Mosaic Virus in Spinach. Phytopathology 87 (9), 924–931.

Yeh, S.-D., Lin, Y.-C., Cheng, Y.-H., Jih, C.-L., Chen, M.-J., and Chen, C.-C., 1992. Identification of tomato spotted wilt-like virus on watermelon in Taiwan. Plant Disease 76, 835–840.

Zhang, L., Handa, K., Palukaitis, P., 1994. Mapping local and systemic symptom determinants of cucumber mosaic cucumovirus in tobacco. The Journal of general virology 75 (Pt 11), 3185–3191.

Zhu, M., Zhang, W.-N., Tian, J.-Y., Zhao, W.-Y., Chen, Z.-Q., Sun, L.-H., Xue, F., Liu, Y., Tan, X.-Q., Wang, L.-M., Liu, F.-Q., Tao, X.-R., 2016. Development of a lateral-flow assay (LFA) for rapid detection of Soybean mosaic virus. Journal of Virological Methods 235, 51–57.

Zilian, E., Maiss, E., 2011. An optimized mRFP-based bimolecular fluorescence complementation system for the detection of protein-protein interactions in planta. Journal of Virological Methods 174 (1-2), 158–165.

7 Abbreviations

%	percent
AGO	Argonaute
ANSV	Alstroemeria necrotic streak virus
AYSV	Alstroemeria yellow spot virus
bp	basepair(s)
BeNMV or BNMV	Bean necrotic mosaic virus
blastn	Basic Local Alignment Search Tool for nucleotides
°C	degree Celsius
CaCV	Capsicum chlorosis virus
CCSV	Calla lily chlorotic spot virus
cDNA	complementary DNA
ChiVMV	*Chilli veinal mottle virus*
CLBV	*Citrus leaf blotch virus*
CMV	*Cucumber mosaic virus*
CP	coat protein
CPsV	*Citrus psorosis virus*
C. jejuni	*Campylobacter jejuni*
C. michiganensis	*Clavibacter michiganensis*
C. sativus	*Cucumis sativus*
CSNV	Chrysanthemum stem necrosis virus
C_t	threshold cycle
CTV	*Citrus tristeza virus*
DAS-ELISA	Double Antibody Sandwich-ELISA
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
DIECA	Diethyldithiocarbamate sodium
DNA	deoxyribonucleic acid
dsDNA/RNA	double-stranded DNA/RNA
DSMZ	Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures
dTTP	deoxythymidine triphosphate
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EPPO	European and Mediterranean Plant Protection Organization
F. fusca, etc.	*Frankliniella fusca*, etc.
Fig.	figure
GBNV	*Groundnut bud necrosis virus*
Gc	glycoprotein (C terminal)
GCFSV	Groundnut chlorotic fan-spot virus

Gn	glycoprotein (N terminal)
GRSV	*Groundnut ringspot virus*
GYSV	*Groundnut yellow spot virus*
hCG	human chorionic gonadotropin
HCRV	Hippeastrum chlorotic ringspot virus
HDA	helicase dependent amplification
INSV	*Impatiens necrotic spot virus*
IYSV	*Iris yellow spot virus*
kb	kilobasepair(s)
kD	kilodalton
kV	kilovolt
LAMP	loop-mediated isothermal amplification
LB	lysogeny broth
LED	light-emitting diode
LFA	lateral flow assay
LFB	lateral flow biosensor
LFD	lateral flow device
LFS	lateral flow strip
LFT	lateral flow test
L.monocytogenes	*Listeria monocytogenes*
LMoV	*Lily mottle virus*
LNRV	Lisianthus necrotic ringspot virus
LOX	lipoxygenase
LSV	*Lily symptomless virus*
M	molar
MFI	median fluorescence intensity
min	minute(s)
mM	millimolar
MP	movement protein
mRNA	messenger RNA
MSMV	Melon severe mosaic virus
MVBaV	Mulberry vein banding-associated virus
MYSV	Melon yellow spot virus
Nad5	mitochondrial NADH dehydrogenase subunit 5
NASBA	nucleic acid sequence based amplification
NASH	Nucleic acid spot hybridization
N. benthamiana	*Nicotiana benthamiana*
NCBI	National Center for Biotechnology Information
NGS	next-generation sequencing
nm	nanometer
N protein	nucleoprotein
ORF	open reading frame
PBST	phosphate buffered saline Tween-20
PCFV	Peanut chlorotic fan-spot virus

PCSV	Pepper chlorotic spot virus
PCR	polymerase chain reaction
pH	potential hydrogen
PhySMV	Physalis severe mottle virus
PLRV	*Potato leafroll virus*
PMTV	*Potato mop-top virus*
PNSV	Pepper necrotic spot virus
PolRSV	*Polygonum ringspot virus*
PPV	*Plum pox virus*
PSTVd	*Potato spindle tuber viroid*
PSV	*Peanut stunt virus*
PVA	*Potato virus A*
PVM	*Potato virus M*
PVS	*Potato virus S*
PVX	*Potato virus X*
PVY	*Potato virus Y*
qPCR	quantitative PCR
RCA	rolling circle amplification
RdRp	RNA-dependent RNA polymerase
RFLP	restriction fragment length polymorphism
RISC	RNA-induced silencing complexes
RNA	ribonucleic acid
RPA	recombinase polymerase amplification
rpm	rounds per minute
R. solanacearum	*Ralstonia solanacearum*
RT-PCR	reverse transcription PCR
s	second(s)
S. aureus	*Staphylococcus aureus*
S. enterica	*Salmonella enterica*
S. lycopersicum	*Solanum lycopersicum*
S. tuberosum	*Solanum tuberosum*
SMV	*Soybean mosaic virus*
sp.	species
spp.	species pluralis
SVNaV	Soybean vein necrosis-associated virus
SVNV	Soybean vein necrosis virus
TAS-ELISA	Triple Antibody Sandwich-ELISA
TASVd	*Tomato apical stunt viroid*
TCDVd	*Tomato chlorotic dwarf viroid*
TCSV	*Tomato chlorotic spot virus*
TMV	*Tobacco mosaic virus*
TNeV	Tomato necrosis virus
TNRV	Tomato necrotic ringspot virus
TNSV	Tomato necrotic spot virus

TPMVd	*Tomato planta macho viroid*
tRNA	transfer RNA
TSPE	target specific primer extension
TSWV	*Tomato spotted wilt virus*
T. tabaci, etc.	*Thrips tabaci*, etc.
TYLCV	*Tomato yellow leaf curl virus*
TYRV	Tomato yellow ring virus
TZSV	Tomato zonate spot virus
U	unit(s)
V. parahaemolyticus	*Vibrio parahaemolyticus*
V. vulnificus	*Vibrio vulnificus*
WBNV	*Watermelon bud necrosis virus*
WSMoV	*Watermelon silver mottle virus*
µM	micromolar
µl	microliter
ZLCV	*Zucchini lethal chlorosis virus*

8 Addendum

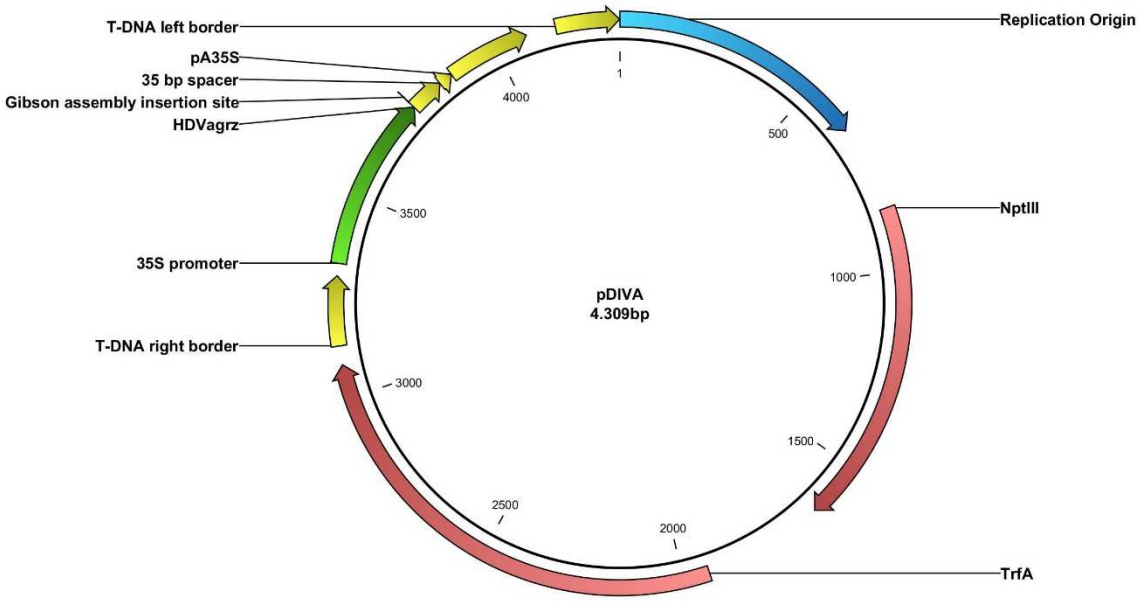

Fig. 8.1 Vector map of pDIVA (accession number KX665539).

Table 8.1 Characteristics of pDIVA vector.

Feature	Description	Position
Replication Origin		1-630
NptIII	Aminoglycoside resistance	842-1636
TrfA	Plasmid replication initiator protein	1935-3083
T-DNA right border		3130-3299
35S promoter	*Cauliflower mosaic virus* 35S RNA promoter	3330-3754
Gibson assembly insertion site	Insertion site for cloned virus RNAs	3755
HDVagrz	*Hepatitis delta virus* antigenomic “core” ribozyme	3755-3839
35 bp spacer		3840-3874
pA35S	*Cauliflower mosaic virus* 35S RNA terminator	3875-4079
T-DNA left border		4153-4309

9 Acknowledgements

Als erstes möchte ich mich bei Prof. Dr. Edgar Maiß für die Möglichkeit bedanken über Pflanzenviren und ihre Nachweismethoden zu promovieren, für seine stetige Unterstützung und auch für sein Verständnis für Elternzeit und Kinderkrankheiten.

Ich danke Prof. Dr. Thomas Debener für die Übernahme des Korreferats und Prof. Dr. Traud Winkelmann für ihre Bereitschaft den Vorsitz der Promotionskommission anzunehmen.

I am grateful to Dr. Jan Bergervoet and Marjanne de Weerdt for their kind introduction into the Luminex xTAG technology and their support during this project.

Bei Prof. Dr. Rainer Blasczyk und Prof. Dr. Stephan Immenschuh bedanke ich mich für die Möglichkeit ihr Luminex 200 System zu nutzen und bei Dr. Eva Zilian, Susanne Aufderbeck und Stefanie Vahlsing für ihre Unterstützung und Hilfe im Umgang mit diesem Messgerät.

Dr. Wulf Menzel und Dr. Stephan Winter danke ich für die Bereitstellung von Virusproben von CMV und Tospoviren.

Ein großes Dankeschön gilt Sarah Trebing für ihre fleißige Mitarbeit am Teilprojekt Volllängenklone und Pseudorekombinanten von CMV, Jutta Zimmermann für ihre Unterstützung bei Klonierungen und Dominik Klinkenbuß, Till Lesker und Hannah Rose für ihre Hilfe mit Rat und Tat und die nette Zeit in Labor und Büro.

Yvonne Koleczek danke ich für ihre stets fröhliche Art und Hilfsbereitschaft, auch bei der Jobsuche.

Seraphine Herrmann, Johannes Specht und Hans-Joachim Seelbinder danke ich für ihre tatkräftige Unterstützung im Gewächshaus bei Anzucht und Pflege der Versuchspflanzen.

Frau Rothenhäuser danke ich für ihre organisatorische Unterstützung.

Allen Mitgliedern der Abteilung Phytomedizin und der AGs Maiß, Reinhard und Boch danke ich für die schöne Zeit am IPP, mit seligen Weihnachtsfeiern, abenteuerlichen Betriebsausflügen und unterhaltsamen Mittagspausen.

Imme Sierk-Krüger danke ich für die herzliche und manchmal sehr spontane Betreuung von Levi und Fred.

Meiner Familie Bald und Blume danke ich für ihre bedingungslose Unterstützung und ihren liebevollen Umgang mit Levi und Fred.

Am Ende möchte ich Linda, Levi und Fred danken. Danke für eure Liebe und die Kraft, die ihr mir schenkt.

10 Curriculum Vitae

Name	Niklas Bald-Blume
Geburtsdatum/-ort	01.10.1984 in Menden

Berufserfahrung

Seit 03.2012	Wissenschaftlicher Mitarbeiter/Doktorand
	Institut für Gartenbauliche Produktionssysteme der Leibniz Universität Hannover
12.2011	Werksstudent
	Institut für Landnutzungssysteme des Leibniz-Zentrums für Agrarlandschaftsforschung (ZALF) in Müncheberg
05.2011 - 07.2011	Praktikant
	Städtische Behörde für natürliche Ressourcen und Umwelt von Lima, Peru

Ausbildung

Seit 03.2012	Promotionsstudium Biologie
	Leibniz Universität Hannover
	angestrebter Abschluss: Dr. rer. nat.
10.2008 - 10.2010	Masterstudium Gartenbauwissenschaften
	Leibniz Universität Hannover
	Abschluss: Master of Science
10.2005 - 09.2008	Bachelorstudium Biologie der Zellen
	Universität Osnabrück
	Abschluss: Bachelor of Science
08.1995 - 07.2004	Walram-Gymnasium Menden
	Abschluss: Allgemeine Hochschulreife
08.1991 – 07.1995	Albert-Schweitzer Grundschule Menden

11 Publication list

Publications without peer review process

1) Bald-Blume, N., Trebing, S., Maiss, E. (2016). Infectious full-length clones of three *Cucumber mosaic virus* isolates with severe symptoms on *Nicotiana benthamiana*. In: Proceedings of the 48th Annual Meeting of the DPG-working group "Virus Diseases of Plants", 07.-08.03.2016, Leibniz Universität Hannover

2) Trebing, S., Bald-Blume, N., Maiss, E. (2016). Creation of a full-length clone of *Cucumber mosaic virus* isolate DSMZ PV-0184 and symptomless infection of *Nicotiana benthamiana*. In: Proceedings of the 48th Annual Meeting of the DPG-working group "Virus Diseases of Plants", 07.-08.03.2016, Leibniz Universität Hannover

3) Bald, N., Bergervoet, J., Maiss, E. (2014). Development of a detection method for plant viruses like tospoviruses and *Cucumber mosaic virus* using the Luminex xTAG® Technology. In: Proceedings of the 59th German Plant Protection Conference, 23.-26.09.2014, Albert-Ludwigs-Universität Freiburg

4) Bald, N., Maiss, E. (2014). Primerdesign for the Luminex xTAG detection of various tospoviruses. In: Proceedings of the 46th Annual Meeting of the DPG-working group "Virus Diseases of Plants", 31.03.-01.04.2014, Julius Kühn-Institut Siebeldingen

5) Bald, N., Bergervoet, J., Maiss, E. (2013). Development of a detection method for Cucumber mosaic virus using the Luminex xTAG® Technology. In: Proceedings of the 45th Annual Meeting of the DPG-working group "Virus Diseases of Plants", 14.-15.10.2013, Julius Kühn-Institut Braunschweig

Bachelor and Master thesis

1) Bald, N. (2010). The relevance of C-terminal regions of the coat protein for symptomatology and aphid transmissability of *Plum pox virus*. Master thesis of the Institute of Plant Diseases and Plant Protection, Leibniz Universität Hannover

2) Bald, N. (2008). Cytological and biochemical investigations about possible functions of the thioredoxin like protein PICOT 1 of *Arabidopsis thaliana*. Bachelor thesis in the department of Plant Physiology, Universität Osnabrück