Molecular and biological investigations for the description and taxonomic classification of celery latent virus and a German *Celery mosaic virus* isolate from Quedlinburg

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Abstract

The *Potyviridae* family, with 195 species and eight genera, is one of the largest families of plant viruses. The members are partly responsible for considerable damage in agriculture, such as the potyvirus *Potato virus Y* (PVY). Nearly all economically important crops are affected by species of this family. Various organisms such as aphids (*Potyvirus*, *Macluravirus*), various mites (*Poacevirus*, *Tritimovirus*, *Rymovirus*) and fungi (*Bymovirus*) serve as vectors of potyvirids. Further transmission modes are mechanically by cultural measures or via seeds. Most viruses belong to the genus *Potyvirus* and their genome consists of a single-stranded positive oriented RNA with a long open reading frame (ORF) encoding a polyprotein comprising ten proteins. Another ORF is embedded in the P3 cistron and expresses an eleventh protein called P3N-PIPO (pretty interesting *Potyviridae* ORF).

In this work, the characterization of two celery-infecting viruses was performed. On the one hand, the celery latent virus (CeLV), whose taxonomic position is still unknown, and a German *Celery mosaic virus* (CeMV) isolate, which is classified into the genus *Potyvirus*, were described.

Since CeLV is associated with the *Potyviridae* due to its particle properties, it does not show pinwheel cytoplasmic inclusion bodies, which are typical for this family so that it is assumed to be an unusual member. A phylogenetic classification of the virus to one of the known genera was not possible, so that CeLV opens a potential new genus with the proposed name celavirus. This fact was confirmed by the calculation of sequence identities to other members of the family. The complete genome sequence showed that CeLV is the largest fully sequenced virus of the *Potyviridae* and has some special features. This includes a very short 5'-UTR combined with the absence of a 3’-poly(A) tail as well as weak sequence matches to other viruses. Using different algorithms, an N-terminal localised signal peptide was predicted and its ability to direct proteins to the endoplasmic reticulum (ER) was confirmed in co-localization studies. An element with this function is not yet described for the *Potyviridae*. Furthermore, a transient local silencing assay was used to limit the presence of a silencing suppressor active region to the first 703 amino acids. The production of an infectious full-length cDNA clone was successful, but the infection rate of two to a maximum of four out of ten *Nicotiana benthamiana* (*N. benthamiana*) was low and the mechanical

transmission to *Chenopodium quinoa* (*C*. *quinoa*) and celery showed the loss of infectivity for *C*. *quinoa*.

Celery mosaic virus from Quedlinburg corresponds to the criteria for the classification as a new isolate and follows the already described separation by geographical origin in the phylogenetic tree. These differences can also be observed in the nucleotide and amino acid sequence. Compared to the only published complete genome sequence of a Californian isolate, a shortened *pipo* is found. The results suggest that the differences are caused by changing conditions in the respective geographical origin. These include environmental conditions such as a change in the composition of potential host plants or vector species. CeMV isolates are either restricted to *Apiaceae* or additionally infect *Solanaceae* or *Chenopodium* species. The molecular background can be investigated in following experiments with the constructed infectious full-length clone.

This work shows the biological and molecular characterization of two viruses in celery with very different characteristics and a first taxonomic classification of CeLV into the family of *Potyviridae* was done.

Keywords: *Potyviridae*, celery latent virus, infectious full-length clone

Zusammenfassung

Die Familie *Potyviridae* zählt mit 195 Spezies und acht Genera zu den größten Familien innerhalb der Pflanzenviren. Die Mitglieder sind teilweise für erhebliche Schäden in der Landwirtschaft verantwortlich, wie zum Beispiel das Potyvirus *Potato virus Y* (PVY). Nahezu alle ökonomisch wichtigen Kulturen werden von Spezies dieser Familie befallen und als Vektoren dienen verschiedene Organismen, wie Blattläuse (*Potyvirus*, *Macluravirus*), Milben (*Poacevirus*, *Tritimovirus*, *Rymovirus*) und Pilze (*Bymovirus*). Weitere Übertragungsmöglichkeiten sind mechanisch durch beispielsweise Kulturmaßnahmen oder über Samen. Die meisten Viren gehören dem Genus *Potyvirus* an und ihr Genom besteht aus einer einzelsträngigen positiv orientierten RNA mit einem langen offenen Leserahmen (ORF), der für ein Polyprotein bestehend aus zehn Proteinen kodiert. Ein weiterer ORF ist im P3 Cistron eingebettet und exprimiert ein elftes Protein mit dem Namen P3N-PIPO (pretty interesting *Potyviridae* ORF).

In dieser Arbeit erfolgte die Charakterisierung von zwei Sellerie-infizierenden Viren. Zum einen handelte es sich um das bisher wenig beschriebene celery latent virus (CeLV), dessen taxonomische Position noch unbekannt ist und ein deutsches *Celery mosaic virus* (CeMV) Isolat, das in das Genus *Potyvirus* eingeordnet wird.

Da CeLV auf Grund seiner Partikeleigenschaften den *Potyviridae* zugeordnet wird, aber keine, für diese Familie typischen, windradförmigen cytoplasmatischen Einschlusskörper zeigt, wird vermutet, dass es sich um ein ungewöhnliches Mitglied handelt. Eine phylogenetische Einordnung des Virus in eines der bisher bekannten Genera war nicht möglich, sodass CeLV ein potentielles neues Genus mit dem Namen celavirus eröffnet. Diese Tatsache wurde durch die Kalkulation von Sequenzidentitäten zu anderen Mitgliedern der Familie bestätigt. Die komplette Genomsequenz ergab, dass es sich um das größte bisher komplett sequenzierte Virus der *Potyviridae* handelt und einige Besonderheiten aufweist. Dazu gehört ein sehr kurzer 5‘-UTR kombiniert mit dem Fehlen eines poly(A) tails sowie schwachen Sequenzübereinstimmungen zu anderen Viren. Durch den Einsatz verschiedener Algorithmen wurde ein N-terminal lokalisiertes Signalpeptid vorhergesagt, dessen Fähigkeit Proteine zum endoplasmatischen Retikulum (ER) zu leiten in Ko-Lokalisationsstudien bestätigt werden konnte. Ein Element mit dieser Funktion ist für die *Potyviridae* bisher nicht

beschrieben. Des Weiteren konnte mit Hilfe eines transienten lokalen Silencing-Assays das Vorhandensein einer Silencing-Suppressor aktiven Region auf die ersten 703 Aminosäuren eingegrenzt werden. Die Erstellung eines infektiösen Volllängenklons war erfolgreich, allerdings ist die Infektionsrate von zwei bis maximal vier von zehn *Nicotiana benthamiana* (*N. benthamiana*) gering und die mechanische Übertragung auf *Chenopodium quinoa* (*C. quinoa*) und Sellerie zeigte den Verlust der Infektiosität für *C. quinoa*.

Das aus Quedlinburg stammende *Celery mosaic virus* entspricht den Kriterien für die Einordnung als neues Isolat und folgt im Stammbaum der schon beschriebenen Auftrennung nach geographischer Herkunft. Diese Unterschiede lassen sich ebenfalls in der Nukleotid- und Aminosäuresequenz feststellen. Im Vergleich zu der einzigen veröffentlichten kompletten Genomsequenz eines kalifornischen Isolates fällt ein verkürztes PIPO Protein auf. Die Ergebnisse legen die Vermutung nahe, dass die Unterschiede durch Veränderungen der jeweiligen geographischen Herkunft bedingt sind. Dazu zählen Umweltbedingungen wie eine veränderte Zusammensetzung von potentiellen Wirtspflanzen oder Vektoren. Weiterhin kann aus der Literatur entnommen werden, dass CeMV Isolate hinsichtlich ihrer Infektiosität entweder auf *Apiaceae* beschränkt sind oder zusätzlich *Solanaceae* oder *Chenopodium* Spezies befallen. Die molekulare Ursache dafür kann durch die erfolgreiche Erstellung eines infektiösen Volllängenklons in folgenden Experimenten ermittelt werden.

Diese Arbeit zeigt die biologische und molekulare Charakterisierung zweier in ihren Eigenschaften sehr unterschiedlichen Viren an Sellerie und eine erste taxonomische Einordnung des bisher wenig beschriebenen CeLV in die Familie der *Potyviridae*.

Schlagworte: *Potyviridae*, celery latent virus, infektiöser Volllängenklon

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1 General Introduction

1.1 The plant virus family *Potyviridae*

1.1.1 Characteristics of the *Potyviridae*

The *Potyviridae* family is one of the largest groups of plant viruses and contains currently eight genera, *Brambyvirus*, *Bymovirus*, *Ipomovirus*, *Macluravirus*, *Poacevirus*, *Potyvirus*, *Rymovirus* and *Tritimovirus*, with 193 approved species and two additional unassigned species, Spartina mottle virus and rose yellow mosaic virus (RYMV). This family is grouped in the picorna-like virus superfamily together with the *Calciviridae*, *Comoviridae*, *Dicistroviridae*, *Picornaviridae* and *Sequiviridae* (Liljas et al. 2002). Two further genera are currently discussed – roymovirus with the unassigned RYMV and bevemovirus with bellflower veinal mottle virus (BVMoV) (Mollov et al. 2013; Seo et al. 2017b, ICTV-pending proposals). About 82 % of the potyvirids belong to the genus *Potyvirus* with its type member *Potato virus Y* (PVY) from which the family and genus name was derived (Wylie et al. 2017).

Worldwide economic damage can be attributed to various *Potyviridae* members infecting a wide range of plants (monocots and dicots) (Urcuqui-Inchima et al. 2001). *Potato virus Y* for example is widespread worldwide and causes massive damage to potatoes and pepper (Budnik et al. 1996; Valkonen 2007). Another prominent representative is *Plum pox virus* (PPV), which is the pathogen of the sharka disease. PPV mainly affects stone fruits and can cause large damage in different *Prunus* crops such as plum and peach (Cambra et al. 2006). Not only the potyviruses are a problem, other genera also have species that cause damage in horticultural and agricultural crops. For example, there is the *Cucumber vein yellowing virus* (CVYV, *Ipomovirus*) in cucumbers and *Barley yellow mosaic virus* (BaYMV, *Bymovirus*) in barley (Cuadrado et al. 2001; Kühne 2009). Symptoms of the above mentioned viruses on leaves and/or fruits are shown in figure 1.1.

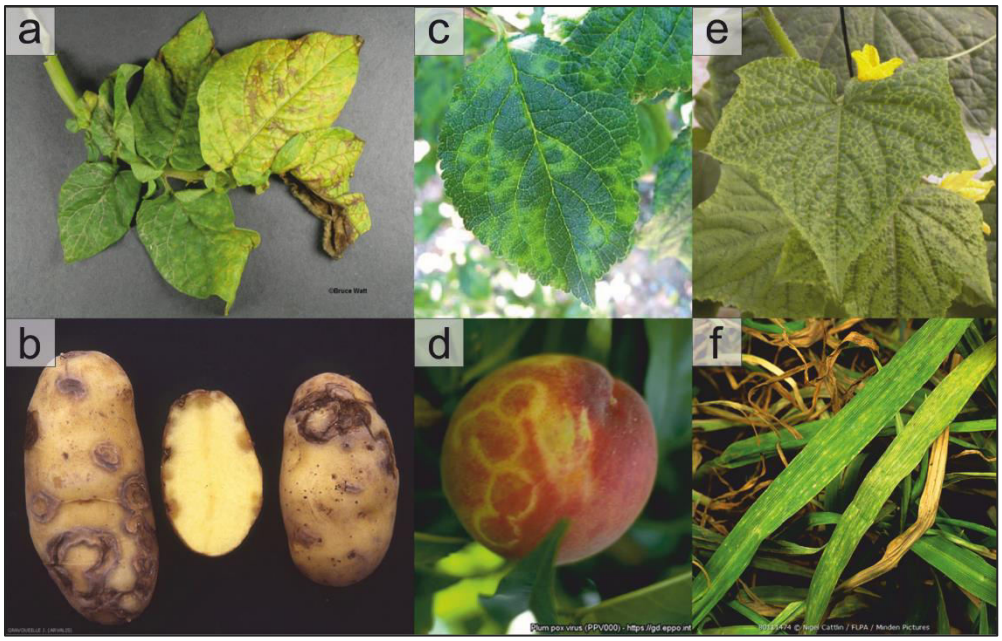

Fig. 1.1: Pictures of leaves and/or fruits infected by different potyvirids. a: PVY on a potato leaf (https://extension.umaine.edu/ipm/wp-content/uploads/sites/3/2010/12/PVY821.jpg, 2017); **b: PVY** on potato tubers (http://ephytia.inra.fr/en/I/30055/Potato-PVYN10, 2017), **c: PPV** on a plum leaf (http://ezramagazine.cornell.edu/FALL08/images/photos/Plum_pox_leaf.jpg, 2017); **d: PPV** on a peach fruit (https://gd.eppo.int/media/data/taxon/P/PPV000/pics/1024x0/1333.jpg, 2017); **e: CVYV** on a cucumber leaf (https://geoplexus.files.wordpress.com/2007/05/cvyv.jpg, 2017); **f: BaYMV** on barley leaves (https://www.mindenpictures.com/cache/pcache2/80111474.jpg, 2017).

All monopartite members within the family form non-enveloped flexuous filamentous particles with a length of 680-900 nm and 11 to 15 nm in diameter (Fig. 1.2 (a)). The virions of the bipartite bymoviruses are 550 and 275 nm respectively. The positive orientated single-stranded RNA ((+)ssRNA) of approximately 10 kilobases (kb) in length is surrounded by 2,000 copies of one coat protein (CP) in a helical symmetry (Dougherty, Carrington 1988; Riechmann et al. 1992). Typical for potyvirids is the formation of pinwheel-like inclusion bodies in the cytoplasm of plant cells by the cylindrical inclusion protein (CI) (Fig. 1.2 (b)) (Dougherty, Hiebert 1980; Sorel et al. 2014). Another possible type of aggregations within the cell are the nuclear inclusions formed by the nuclear inclusion protein a (NIa) and nuclear inclusion protein b (NIb) and there are also reports for amorphous inclusions made up of the helper component-protease (HC-Pro). Instead of the pinwheels, the formation of the other inclusions can vary between the species (Riechmann et al. 1992).

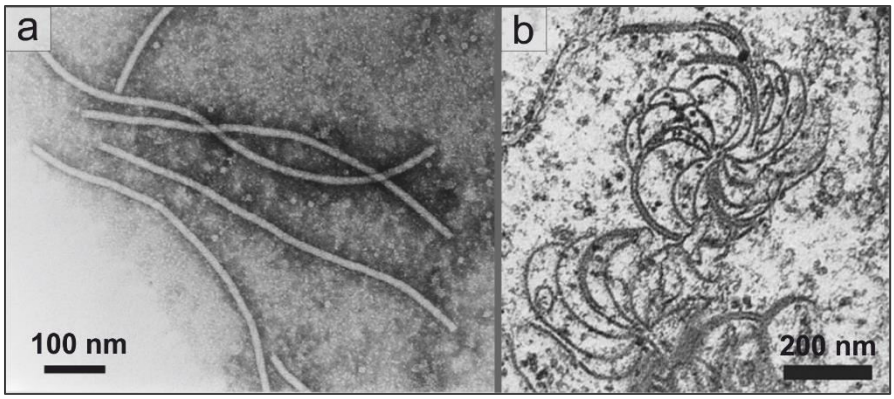

Fig. 1.2: Electron micrographs of *Potato virus Y* particles (a) and cylindrical pinwheel inclusions (b).
a: http://www.dpvweb.net/dpvfigs/a1.jpg, 2017, modified;
b: http://www.dpvweb.net/dpv/showfig.php?dpvno=37&figno=08, 2017, modified

Members of the family are classified by several characteristics as host range, vector transmission, serology and, since sequences are available by comparisons of nucleotides and amino acids of cistrons, proteins or the whole genome/polyprotein (Adams et al. 2005b).

1.1.2 Genome organization and expression strategy of potyviruses

The *Potyvirus* genus with its 160 species is the largest within the *Potyviridae*. In the following chapters 1.1.2 to 1.1.4, the characteristics of the family are presented using the genus *Potyvirus* as an example. The other genera with their differences to potyviruses are described in chapter 1.1.5.

The potyviral genome has a viral protein genome-linked covalently bound to the 5'-end of the (+)ssRNA followed by an untranslated region (UTR) and the large open reading frame (ORF). The 3'-end consists of an additional UTR and is polyadenylated (Maiss et al. 1989; Riechmann et al. 1989). The genome size is about 10,000 nts and encodes a single long open reading frame from which one large polyprotein is translated (Fig. 1.3).

Due to the lack of a 5'-cap structure the translation has to be performed cap independent. The 5'-UTR of *Tobacco etch virus* (TEV) was found out to enhance the translation and mimic the function of an internal ribosome entry site (IRES) (Carrington, Freed 1990). Together with the poly(A) tail, an efficient translation is performed (Gallie et al. 1995). The PPV 5'-UTR differs from this organization and does not contain the IRES-like elements but it could be detected that a leaky-scanning mechanism is involved in translation. The covalently bound viral protein genome-linked

(VPg) is not essential for translation but it was shown to recruit the translation initiation factor 4E (eIF4E) of the host cell and subsequently the poly(A) binding protein (PABP) which interacts with the viral poly(A) tail (Kneller et al. 2006).

During translation, the polyprotein is cleaved into ten individual proteins by three viral encoded proteases. The first protein is P1, followed by the HC-Pro, P3, 6K1, CI, 6K2, NIa-VPg, NIa-Pro, NIb and CP (Urcuqui-Inchima et al. 2001). A second smaller ORF was discovered within the P3 cistron and called (pretty interesting *Potyviridae* ORF (*pipo*). The ORF is translated via a ribosomal +2 frameshift at a highly conserved motif $G_{1\text{-}2}A_{6\text{-}7}$ in the N-terminal region leading to a fusion protein consisting of the P3 N-terminus and PIPO (P3N-PIPO) (Chung et al. 2008; Olspert et al. 2015).

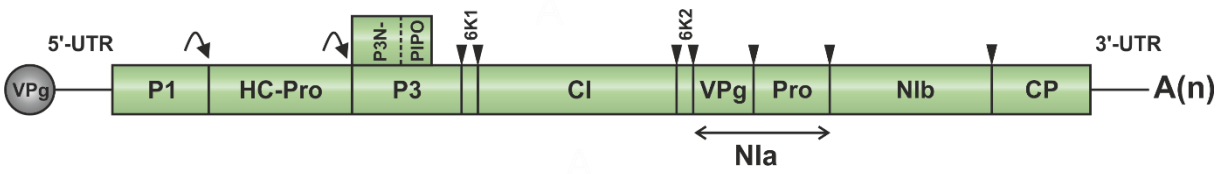

Fig. 1.3: Schematic overview of the genome organization of potyviruses. The RNA is illustrated in form of a black line and the long ORF as a box and separated into the different cistrons. The grey filled circle displays the VPg attached to the 5'-end. Curved arrows above P1 and HC-Pro indicate the self-cleavage of P1 and HC-Pro and black filled triangles mark the cleavage sites of the NIa-Pro. For abbreviations see explanations in the text.

The polyprotein is processed by three viral proteases, whereby P1 and HC-Pro release themselves at the respective C-terminus. All other proteins are released by cleavage from the NIa-Pro (Carrington et al. 1989; Merits et al. 2002).

1.1.3 Replication, movement and transmission in the plant cell

After entering a plant cell, the virus uncoating begins and the RNA is released in the cytoplasm. There, it is translated to generate the NIb which is the key protein in the replication. The replication starts by synthesizing a complementary negative RNA strand which is used as a template for new positive strands (Hull 2014). In a next step, initiating the formation of the viral replication complex (VRC) at the endoplasmic reticulum (ER) membrane with the help of the integral membrane protein 6K2 takes place from that later on vesicles are guided to the chloroplasts. VPg is thought to be linked to the 6K2, has a nucleotide binding capacity and is essential for replication by possibly serving as a priming element (Murphy et al. 1996; Schaad et al. 1997a; Puustinen, Mäkinen 2004; Wei et al. 2010a). The viral RNA is synthesized by the NIb

with its function as an RdRp and the emerging double-stranded intermediates are separated by the CI which acts as a helicase (Domier et al. 1987; Laín et al. 1989). The NIa-Pro is able to bind RNA and was shown to interact with NIb possibly directing it to the replication complex (Merits et al. 2002). It was recently shown, that 6K1 is involved in the early replication steps and could have a potential role in the VRC assembly (Cui, Wang 2016). Further studies revealed that P3 is also essential for replication and that the C-terminus is responsible for targeting P3 to the VRC (Cui et al. 2017). To date the detailed steps and functions of involved proteins are still not completely understood.

In order to reach adjacent cells and establish an infection, the viral RNA has to be transported across the cell borders. The plasmodesmata across the cell-wall serve as a connection between neighbouring cells and are used by viruses to move into the next cell (Maule 2008). Potyviruses do not encode a single movement protein (MP) but several proteins are involved in the short-distance movement as for example the CI, which binds the virions. P3N-PIPO binds CI in this complex and guides it to the plasma membrane by binding PCaP1, an integral membrane protein. The complex is then translocated to the plasmodesmata where CI forms a filamentous structure (Wei et al. 2010b; Vijayapalani et al. 2012). To pass through, the size exclusion limit has to be increased and this is potentially managed by the CP and HC-Pro (Rojas et al. 1997).

To infect the whole plant, potyvirids have to reach the sieve elements in the phloem and to be transported through them. To establish a new infection, the viruses then have to exit the sieve cells again to get to the cytoplasm of non-infected cells. Several viral proteins are involved in this steps. It could be shown, that the N- and C-termini of the CP play an essential role in long-distance movement (Dolja et al. 1995). Due to the high variations in the CP N-terminus, it is thought that probably the charge is more important than the primary sequence (Kimalov et al. 2004). The VPg is also involved and it is speculated whether CP and VPg are parts of a movement complex (Schaad et al. 1997b; Revers et al. 1999). The detailed function of 6K2 in this context is still not completely understood but a possible anchorage of the movement complex to ER membranes is discussed (Spetz, Valkonen 2004). Other studies revealed the presence of VPg in phloem-associated cells in sink leaves, possibly being involved in virus unloading (Rajamäki, Valkonen 2003). To enter and exit the plant vascular system the

HC-Pro could possibly be needed but the detailed functions are not known (Cronin et al. 1995).

To move from plant to plant, viruses are dependent on either a mechanical transmission, vectors, seeds and/or pollen. In the genus *Potyvirus*, there are some members which are seed transmissible and most members can infect plants after a mechanical treatment (Khetarpal, Maury 1987; Singh et al. 2005). Potyviruses are predominantly vectored by aphids in a non-persistent manner with short times of acquisition and retention. When the aphids feed on an infected plant, the virus and HC-Pro proteins are taken up and during this stage, the HC-Pros form dimers which function as a "bridge" by binding the stylet at a yet not identified protein/receptor and the CP via conserved binding sites (Ng, Falk 2006; Blanc et al. 2014). Feeding on a healthy plant then leads to the release of the virus by salivary secretions after puncturing a cell (Martín et al. 1997).

1.1.4 The functions of potyviral proteins

Potyviral proteins have been shown to be multifunctional and in the following passages an overview of the most important functions is given.

P1

P1 is one of the least conserved proteins and acts as a trypsin-like serine protease whose active centre consists of the amino acids histidine, aspartic acid and serine. The catalytic centre is located in the C-terminal part of the protein and the active serine is embedded in a highly conserved motif G-X-S-G (Bazan, Fletterick 1988; Adams et al. 2005a; Valli et al. 2007). The cleavage site is 22 to 28 amino acids downstream of a conserved R-G motif and consists mostly of a phenylalanine or tyrosine at the P1 position and serines at the P1' position (Adams et al. 2005a). To date, the exact functions of P1 have not yet been fully characterized. However, other properties can be assigned to it. It has been shown that P1 is not absolutely necessary for the replication cycle (Rohožková, Navrátil 2011). It binds RNA in an unspecific manner and enhances the activity of HC-Pro (Brantley, Hunt 1993; Pruss 1997). Furthermore, it is believed to be involved in genome amplification and has an influence on the host plant spectrum (Verchot, Carrington 1995; Salvador et al. 2008b). Some P1 proteins contain zinc-finger motifs, such as *Pea seed-borne mosaic virus* (PSbMV) but the detailed function is still unknown (Rohozková et al. 2014; Valli et al. 2007).

Helper component-protease (HC-Pro)

The HC-Pro can be divided into three regions: N-terminal, central and C-terminal part. It is the second encoded protease, a cysteine-protease that cleaves itself autocatalytically between a glycine dipeptide (Carrington et al. 1989). The catalytic diad is composed of cysteine and histidine located in the C-terminal part (Oh, Carrington 1989). Additionally, the HC-Pro is involved in aphid transmission and acts as a bridge between the viral CP and the stylet of the vector. Two amino acid motifs in the HC-Pro were identified and proved to be essential for this purpose. The C-terminal located P-T-K motif interacts with the CP and the N-terminal located K-I-T-C binds to the stylet of the aphid (Atreya, Pirone 1993; Huet et al. 1994; Blanc et al. 1997). Studies to identify the exact interaction partner(s) of HC-Pro failed but there are several cuticle proteins suspected (Dombrovsky et al. 2007). A few years later other possible interaction partners of HC-Pro were found and a direct interaction with a ribosomal protein S2 was confirmed. This protein shows homologies to the laminin receptor precursor, which is considered to be a receptor for other viruses like alphaviruses in mammalian cells (Nelson et al. 2008; Fernández-Calvino et al. 2010).

Furthermore, the HC-Pro was the first silencing suppressor to be described (Anandalakshmi et al. 1998). There are many ways how the plant defence mechanism is interfered. For TEV, it has been shown that HC-Pro binds the viral siRNAs via a central located conserved F-R-N-K motif thereby removing them from the RNA interference (RNAi) mechanism (Lakatos et al. 2004; Shiboleth et al. 2007). Other possibilities are inhibition of the methyltransferase HEN1 that modify siRNAs or the interaction with argonaute 1 (AGO1) (Ivanov et al. 2016). There are indications, that the HC-Pro is involved in the viral movement and genome amplification (Cronin et al. 1995; Rojas et al. 1997).

P3 & pretty interesting *Potyviridae* ORF (PIPO)

Until today, the P3 functions are still not completely understood. It was speculated, if P3 is involved in symptomatology and serves as a pathogenicity determinant (Sáenz et al. 2000; Suehiro et al. 2004). A potential assignment in replication was recently shown in which the C-terminus of P3 is responsible for targeting P3 to the VRC (Cui et al. 2017).

Chung et al. discovered a small ORF within the coding region of the P3 N-terminus and named it pretty interesting *Potyviridae* ORF (*pipo*). This protein is translated by a ribosomal +2 frameshift in a special conserved slippery sequence $G_{1-2}A_{6-7}$. It was shown that P3N-PIPO is involved in viral movement (Chung et al. 2008). It guides CI (bound to the virion) to the plasma membrane by binding PCaP1, an integral membrane protein, translocating the complex to the plasmodesmata where CI forms a filamentous structure (Wei et al. 2010b; Vijayapalani et al. 2012).

6K1 & 6K2

So far, no clear functions could be assigned to the 6K1 except for the assumption that it plays a role in infectivity because a deletion led to non-infectious *Potato virus Y* (Merits et al. 2002). This fact was supported by the studies of Cui et al., in 2016. They could prove that 6K1 is involved in replication and probably plays a role in the assembly of the VRC (Cui, Wang 2016).

The second 6K protein plays a regulatory role in the genome-replication. When bound to NIa, the latter is not possible to enter the nucleus (Restrepo-Hartwig, Carrington 1992). Apart from this, the 6K2 is an integral membrane protein and induces the formation of ER-derived vesicles for the VRC which are translocated to the chloroplasts (Schaad et al. 1997a; Wei et al. 2010a).

Cylindrical inclusion protein (CI)

The CI is a helicase classified into the superfamily 2 and related to pesti- and flaviviruses (Laín et al. 1989). It is contains several conserved motifs with different functions like nucleoside-triphosphatase (NTPase) and helicase (Laín et al. 1989; Fernández et al. 1995). CI has the ability to target the plasmodesmata and interact with itself and P3N-PIPO to support the viral short-distance movement (Wei et al. 2010b; Zilian, Maiss 2011; Vijayapalani et al. 2012). Due to the self-interaction, the characteristic pinwheel cytoplasmic inclusion bodies are formed which are located near the plasmodesmata (Sorel et al. 2014).

Viral protein genome-linked (VPg)

VPg is the N-terminal domain of the NIa and covalently bound to the 5'-end of the viral RNA. It has the ability to recruit the translation initiation factor 4E (eIF4E) of the host cell and subsequently the poly(A) binding protein (PABP) which interacts with the viral

poly(A) tail (Kneller et al. 2006). If the VPg is not able to bind to the 5'-end, replication will no longer take place, indicating that it is essential for this function (Murphy et al. 1996). Other functions are involvement in long-distance movement in the early stages on infection by uploading the virus into the sieve cells and its acting as a host determinant (Schaad et al. 1997b; Rajamäki, Valkonen 2003). By comprising two nuclear localization signals (NLSs), the protein is translocated into the cell nucleus and forms characteristic nuclear inclusion bodies of the unprocessed form of NIa (Rajamäki, Valkonen 2009).

Nuclear inclusion protein a (NIa)

The NIa-Pro, categorized as trypsin like protease with cysteine instead of serine in the catalytic centre, is responsible for the release of most of the functional proteins from the potyviral polyprotein. It can process in both, cis and trans, and the catalytic residues are histidine, aspartic acid, cysteine and an additional histidine (Carrington, Dougherty 1987; Merits et al. 2002; Adams et al. 2005a). The cleavage sites can slightly vary between viruses and genera and the most common patterns for the positions P4 to P1' are (V,I)-(E,R,I)-(G,F,L)-(Q,E)/(S,A,G) (Adams et al. 2005a). Despite of its function as a protease, NIa shows unspecific desoxyribonuclease (DNase) activity and interacts with the NIb playing a potential role in the replication (Merits et al. 2002; Anindya, Savithri 2004).

Nuclear inclusion protein b (NIb)

The NIb functions as an RdRp and is the main actor in genome amplification. Its active centre is highly conserved and embedded in a G-D-D motif (Hong, Hunt 1996). Studies revealed that NIb carries two independent NLSs and is translocated into the nucleus where it forms nuclear inclusions (Li et al. 1997).

Coat protein (CP)

The coat protein can be divided into three domains: N-terminal, central and C-terminal region whereof N- and C-terminus are exposed to the surface. Around 2,000 copies of the CP encapsidate one RNA molecule (Dougherty, Carrington 1988). Furthermore, it is involved in aphid transmission. It could be shown, that a highly conserved motif in the N-terminus (D,N)-A-G is responsible to interact with the HC-Pro and mutations abolished this feature (Atreya et al. 1995; Blanc et al. 1997). In order to spread within

the plant, the virus has to be transported in short- and long-distance terms in which the CP plays a crucial role (Dolja et al. 1995; Rojas et al. 1997).

1.1.5 Description of the other genera and unassigned viruses

In this paragraph a brief overview of the other genera in the *Potyviridae* is given. A schematic figure illustrating the genomes is presented in figure 1.4 (Revers, García 2015).

Brambyvirus

The genus *Brambyvirus* comprises one species, the *Blackberry virus Y* (BlVY), a *Rubus* infecting virus (Carstens 2010). The name *Brambyvirus* is derived from the host's name bramble. The virus was first described in 2008 by Susaimuthu et al. and shows in general the same genomic elements as potyviruses (Susaimuthu et al. 2008). With its genome size of 10,851 nts it was the largest fully sequenced member of the *Potyviridae* at that time. The reasons for the classification into a new genus are mainly sequence variations in the N-terminal area of the polyprotein. The BlVY has an unusually large P1 (745 aa) and a smaller HC-Pro. The N-terminus of P1 contains an AlkB domain, which has not been described for potyvirids before. AlkB and homologous domains are responsible for repairing DNA and RNA from alkylation damage (Aas et al. 2003). Studies on plant viral AlkB domains of *Grapevine virus A* (GVA, *Flexiviridae*), *Blueberry scorch virus* (BlScV, *Flexiviridae*) and BlVY showed a similar way of action and it has been speculated whether the AlkB domain is involved in silencing suppression by a yet unknown mechanism (van den Born et al. 2008). BlVY HC-Pro is about 120 amino acids shorter than that of potyviruses. The missing part conforms to the N-terminus of potyviral HC-Pros, which is probably responsible for vector transmission and silencing suppression. In addition, the protein lacks motives for genome amplification and systemic movement. To date the vector is still unknown and there is no other species that could be classified to this genus.

Bymovirus

The bymoviruses are the only bipartite members of the family with currently six species. The type member is *Barley yellow mosaic virus* (BaYMV) which was name-giving for the genus (Wylie et al. 2017). Both RNAs are packaged individually and have a VPg covalently bound to the 5'-end. RNA1 encodes the region from P3 to CP and has an approximate size of 7.6 kb and RNA2 encodes P1 and P2 and is 3.6 kb in size (Usugi

et al. 1989; Kashiwazaki et al. 1990; Kashiwazaki et al. 1991). The particles are between 550 and 275 nm respectively. P1 is a cysteine-like protease and considered to cleave the polyprotein encoded by RNA2 into P1 and P2 (Adams et al. 2005a). P2 is believed to be involved in vector transmission, other functions are not yet known. The bymoviral CI is flanked by two 7K proteins (*Rice necrosis mosaic virus*, RNMV) or a 7K and a 14K protein (*Oat necrotic mottle virus*, ONMV) instead of the common two 6K proteins of potyviruses (Kashiwazaki 1996; Wagh et al. 2016). The vectors of bymoviruses are members of the *Plasmodiophorales* family - the soil-born *Polymyxa graminis* (Davidson et al. 1991). Their host plant spectrum is limited to the *Poaceae* such as wheat, rice and barley.

Ipomovirus

The *Ipomovirus* genus has currently six species whereof *Sweet potato mild mottle virus* (SPMMV) is the type member. It was first discovered in sweet potato (*Ipomea* sp.) and is transmitted by the whitefly *Bemisia tabaci* in a semi-persistent manner (Harpaz, Cohen 1965; Colinet et al. 1998; Maruthi et al. 2005; Adkins et al. 2007; Abraham et al. 2012). Ipomoviruses occur worldwide and can infect numerous plants from different families like *Apiacae*, *Cucurbitaceae*, *Convolvulaceae* and *Solanaceae* (Morris et al. 2006; Dombrovsky et al. 2014). Within the genus the genome organization varies in the N-terminal region of the polyprotein. SPMMV shows the typical cistrons like the potyviruses but it was shown, that the large P1 instead of HC-Pro is the silencing suppressor owning N-terminal located W-G/G-W motifs, possibly being responsible for Agronaute (AGO) binding (Giner et al. 2010). *Tomato mild mottle virus* (TMMoV) shows the same genomic organisation but the silencing suppressor was not yet studied (Abraham et al. 2012). *Squash vein yellowing virus* (SqVYV) and *Cucumber vein yellowing virus* (CVYV*)* do not encode a HC-Pro but instead a doubled P1 - P1a and P1b - of which P1b is assumed to be involved in silencing suppression (Valli et al. 2006). Another variant of the genome composition is found in the genomes *Cassava brown streak virus* (CBSV) and *Ugandan cassava brown streak virus* (UCBSV). They do not encode for either an HC-Pro or a P1b, but have a P1 which is directly followed by the P3. In this case, the silencing suppressor is the P1. Another special feature of these two species is a HAM1 like sequence between the NIb and the CP. Due to homologies to cellular Maf/HAM1 NTP pyrophosphatases it is speculated,

that this domain reduces the mutation rates by interfering with noncanonical nucleoside triphosphates (NTPs) (Mbanzibwa et al. 2009).

Macluravirus

The genus *Macluravirus* contains eight species and harbours the smallest potyvirids within the family. The reason for that is that they do not encode a P1 and their genome starts directly with a HC-Pro (Kondo, Fujita 2012). This could be the reason for the occurrence of slightly smaller particles of 650 to 675 nm compared to potyviruses with a size over 680 nm (Wylie et al. 2017). The name-giving type member *Maclura mosaic virus* (MacMV) was responsible for symptoms on the ornamental tree *Maclura pomifera* and the formation of cylindrical cytoplasmic inclusions (Badge et al. 1997; Pleše, Miličić 1973). The HC-Pro as the first protein in the macluraviral polyprotein is about 200 amino acids smaller than in potyviruses and lacks the regions responsible for silencing suppression, systemic movement and aphid transmission. Nevertheless, aphid transmission is performed effectively (Kondo, Fujita 2012; Minutillo et al. 2015; Zhang et al. 2016).

Poacevirus

The genus is composed of three species and was named after the natural hosts of the first two species: *Poaceae* like wheat and sugarcane (Hema et al. 2002; Seifers et al. 2008). Their genome shows the “common” potyviral cistrons. *Sugarcane streak mosaic virus* (SCSMV) and *Wheat streak mosaic virus* (WSMV) were initially proposed to be a member of one putative genus (Hall et al. 1998). WSMV was then the first member of a new genus *Tritimovirus* and SCSMV as a possible one (Stenger et al. 1998). A few years later, for SCSMV a new genus susmovirus was suggested with SCSMV as the type member (Viswanathan et al. 2008). In 2009 it was found that a new potyvirid, *Triticum mosaic virus* (TriMV), is a distinct member and forms a new genus with the proposed name *Poacevirus* together with SCSMV (Tatineni et al. 2012). The third species, *Caladenia virus A*, was isolated from plants of the *Orchidaceae* thus, being the first member of a host not belonging to the *Poaceae* (Wylie et al. 2012). TriMV is transmitted by the wheat curl mite (*Aceria tosichella*), the vector of the other members is still unknown (Seifers et al. 2009).

Rymovirus

The genus *Rymovirus* belongs to one of the first approved genera in the newly established family *Potyviridae* set up after the *Potyvirus* workshop of the *Potyvirus* Study Group in 1990. The type species *Ryegrass mosaic virus* (RGMV) is transmitted by the cereal rust mite (*Abacarus hystrix*) (Mackenzie et al. 1999). Rymoviruses show a close relationship to the genus *Potyvirus* and the existence of the genus was/is questioned. Sequence comparisons and phylogenetic analyses of the three members *Agropyron mosaic virus* (AgMV), *Hordeum mosaic virus* (HoMV) and RGMV reveal on the one hand exceeding thresholds for genus demarcation and on the other hand the formation of a distinct clade (Adams et al. 2005b; French, Stenger 2005; Ward 2017). It is assumed, that the potyviruses evolved from the rymoviruses (Gibbs, Ohshima 2010).

Tritimovirus

The name of the genus *Tritimovirus* was derived from the host *Triticum* sp. and the mosaic symptoms which are caused by its type member WSMV (Stenger et al. 1998). The host range is restricted to *Poaceae* and transmission is performed by the wheat curl mite *Aceria tosichella* (Stephan et al. 2008). Despite of being an additional genus which is transmissible by mites, the viruses form a distinct cluster in the phylogenetic tree (Wylie et al. 2017). In different experiments it could be shown, that the HC-Pro of WSMV is involved in mite-transmission (Stenger et al. 2005). By mutation of conserved cysteines (aa 16, 46 and 49) in a zinc-finger-like motif in the N terminal region, vector transmission was not possible anymore (Young et al. 2007).

Unassigned

It was shown that Spartina mottle virus (SpMV) had a serological relationship to AgMV, which was a member of the genus *Potyvirus* at that time and based on this characteristics, SpMV was also classified as a member of this genus (Jones 1980). Sequence and phylogenetic analyses of the complete genome sequence (Maiss pers. comm.) revealed that SPMV shows the typical potyviral genome organization but could not be classified into the known genera so far, supporting the previous proposal of a new genus sparmovirus (Götz et al. 2002). The vector is still unknown.

Rose yellow mosaic virus (RYMV) encodes the same genomic composition and like SPMV, on the basis of sequence comparisons and phylogenetic analyses, it is not possible to classify it to one of the known genera. RYMV clusters near to tritimo- and poaceviruses, so far a vector is unknown (Mollov et al. 2013).

Schematic overview of the genome organization

The following figure illustrates the genomes of several (type) members of the different genera within the *Potyviridae* (Revers, García 2015).

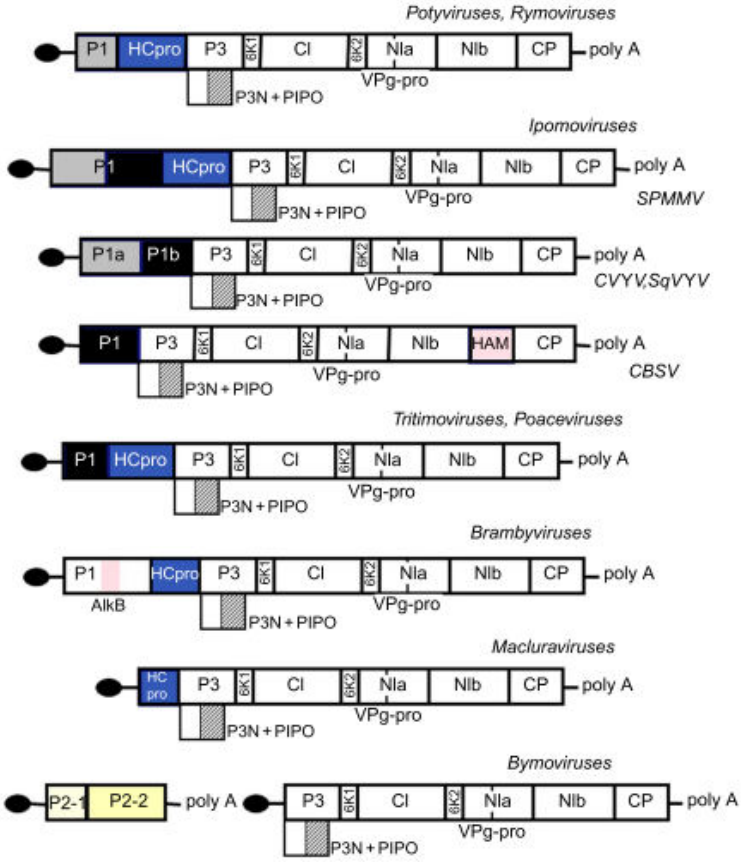

Fig. 1.4: Schematic overview of the genome organization of (type) members of the different genera within the *Potyviridae*. The long ORF is shown as a box and separated into the different cistrons and the black ellipse illustrates the VPg attached to the 5'-end. The boxes below the P3 & striped parts mark P3N-PIPO & PIPO. The HC Pros are displayed as blue elements. The P1s are separated into subgroups: Grey: *Potyvirus*-like P1s; black: P1b-like P1s; yellow & light yellow: bymoviral RNA2 encoded products, P1: P2-1, P2: P2-2. Other domains like AlkB and HAM are coloured in pink. The picture is taken from Revers, García 2015.

1.2 *Apium* infecting viruses

Apium graveolens species belong to the family *Apiaceae* (or *Umbelliferae*) which contains about 434 genera and 3,700 species. Other members are for example carrot (*Daucus carota*), fennel (*Foeniculum vulgare*) or dill (*Anethum graveolens*) (Stevens, Davis 2005). In Germany the cultivation area for celery (*Apium graveolens* var. *dulce*) and celeriac (*Apium graveolens* var. *rapaceum*) was 278 and 1,667 ha respectively in the year 2016 (Federal Statistical Office, Destatis, 2016). Australia grows celery on an area of 1,695 ha in the year 2015 (The Australian Statistics Handbook 2015/2016). The USA are one of the biggest growers with approximately 11,700 ha in the year 2016 (vegetable summary report, USDA, 2017). Celery and celeriac are affected by several viruses from different genera and families. From the *Bromoviridae*, there are for example *Cucumber mosaic virus* (CMV, *Cucumovirus*) as well as *Alfalfa mosaic virus* (AMV, *Alfamovirus*). The *Secoviridae* are represented by *Celery yellow vein virus* (CYVV, serotype of *Tomato black ring virus*) and *Strawberry latent ringspot virus* (SLRSV, unassigned genus) and the *Bunyaviridae* with *Tomato spotted wilt virus* (TSWV, *Tospovirus*) (Hollings 1965; Walkey, Mitchell 1969; Pemberton, Frost 1986; Li et al. 2015). In addition, several potyviruses were reported in *A. graveolens* like *Angelica virus Y* (AnVY), *Arracacha mottle virus* (ArMV), *Apium virus Y* (ApVY), *Panax virus Y* (PanVY) and *Celery mosaic virus* (CeMV) (Severin, Freitag 1938; Robertson 2007; Orílio et al. 2009; Yan et al. 2010; Xu et al. 2011a). The symptoms range from vein clearing, mosaics, necrotic rings and spots, leaf curling, stunting and reduced plant growth.

A virus, which does not cause symptoms in celery was discovered in Italy and named celery latent virus (Brandes, Luisoni 1966). It has not yet been classified but virus particles resemble *Potyvirus* particles. CeMV and CeLV will be described in detail in the next chapters because they are the research subjects of this work.

1.2.1 Celery latent virus (CeLV)

CeLV was first described by Brandes and Luisoni in 1966. It was discovered in a symptomatic plant in Italy but in experimental infection of *Apium* sp. the original symptoms could not be reproduced, indicating a mixed infection with another virus or other factors. As the name says, there were mostly no or very faint symptoms occurring, when transmitted to *Apium* sp. (Brandes, Luisoni 1966). In a further

characterization by Bos et al. 1977, experimental hosts such as *Spinacia oleracea*, *Nicotiana megalosiphon* (latent systemic), *Pisum sativum* (systemic) as well as the symptom developments were investigated. The virus causes local and systemic mosaic symptoms in *C. quinoa* and it is transmissible in 67 % by seeds. In *Apium* sp. the seed transmission rate was 34 %. With regard to the transmission by animal vectors, for example insects, five aphid species have been tested to date (among others: *Aphis fabae* and *Myzus persicae*) but none of them was able to transmit the virus to a healthy plant. In electron microscopic investigations of *Tobacco mosaic virus*- (TMV) and CeLV-infected *Chenopodium* species, flexible filamentous particles with a length of 885 nm for CeLV could be determined. The particles show similarities to viruses from the *Potyviridae* family. Unlike potyviruses, no pinwheel-like structures have been observed. The economic impact of this virus is questionable (Bos et al. 1978). So far, no sequence data or a taxonomic classification were published.

1.2.2 *Celery mosaic virus* (CeMV)

The first reports about the celery mosaic disease occurred 1922 in New Jersey and reports about *Celery mosaic virus* 1935 in California (Poole 1922; Severin, Freitag 1938). At that time, celery was already known to contain a virus called *Southern celery mosaic virus*. *Celery mosaic virus* differed in terms of symptomatology and was similarly referred to as *Western celery mosaic virus*. Brandes and Luisoni described a virus with similar characteristics and suggested in 1966 that the addition "western" should be dispensed and *Celery mosaic virus* the species name (Brandes, Luisoni 1966). To date the virus occurs worldwide with reports from England (Pemberton, Frost 1974), Australia (Latham, Jones 2003), Netherlands (Bos et al. 1989), Germany (Brandes, Luisoni 1966), Venezuela (Fernández et al. 1995), Poland (Paduch-Cichal, Sala-Rejczak 2010), Iran (Khoshkhatti et al. 2011) and Egypt (Amal et al. 2012). In Australia and the USA CeMV epidemics led to massive economic damage in the celery production. The introduction of a celery-free period of about three months was able to reduce the problem (Severin, Freitag 1938; Latham, Jones 2003). The virus belongs to the genus *Potyvirus* and shows the typical genome organization (Xu et al. 2011b). To date one complete genome of a Californian isolate (NC_015393.1) is available from the National Centre for Biotechnology database (NCBI) and several partial sequences from different countries.

1.3 Infectious full-length cDNA clones of plant viruses

Infectious full-length cDNA clones of plant virus genomes offer lots of advances and possibilities in virus research. Experiments with a full-length cDNA clone start with one distinct sequence and not with a virus population in which slight sequence variations are possible. Full-length clones offer the possibility to study viral protein functions by insertion of mutations, creating chimeric viruses, investigating complementation and trace the movement of viruses by labelling with a reporter gene (Boyer, Haenni 1994). To date, about 150 full-length sequences of potyvirids are deposited in the NCBI database and of more than 25 species infectious full-length cDNA clones are available. The infection of the plant is either performed via particle-bombardment of DNA and RNA, inoculation of RNA transcripts after *in vitro* transcription or *R*. *radiobacter* mediated infiltration. Some examples for full-length clones of potyviruses are PVY (Jakab et al. 1997), PPV (Maiss et al. 1992) and *Soybean mosaic virus* (SMV) (Seo et al. 2009). From other genera there are for example *Chinese yam necrotic mosaic virus* (CYNMV, *Macluravirus*) (Kondo, Fujita 2012) WSMV (*Tritimovirus*) (Choi et al. 1999) and BaYMV (*Bymovirus*) (You, Shirako 2010) reported.

For study of distinct protein functions for example motifs involved in aphid transmission were investigated. In *Tobacco vein mottling virus* (TVMV) mutations in a full-length clone were introduced into the K-I-T-C motif (HC-Pro) exchanging the K by other amino acids (Atreya, Pirone 1993). Similar experiments were done to characterize the D-A-G motif in the CP of TVMV (Atreya et al. 1990). Dietrich et al. labelled two full-length clones of PPV with dsRed and GFP and constructed chimeric viruses to investigate recombination events in the 3'-end of the genome. With the help of a confocal laser scanning microscope the spread within the plant could be observed for mixed infection of the chimera with the wild-type virus (Dietrich et al. 2007). Cloning of viral RNAs is challenging due to their large size of about 10 kb. There are several methods available to clone fragments, like the use of sticky ends produced by restriction endonucleases with subsequent ligation (Maiss et al. 1992) or with a technique named Gibson Assembly (Bordat et al. 2015).

1.4 Objectives

The *Potyviridae* is one of the largest plant virus family with eight genera and 195 species, of which 160 belong to the genus *Potyvirus*. The viruses are transmitted by numerous vectors such as aphids, whiteflies, fungi or mites. Within the family, there are sometimes considerable differences in the genome organisation and protein function between and within the genera. The number of species and sequence data continues to increase and two additional genera are currently being discussed by the ICTV. *A*. *graveolens* varieties are infected by many viruses, including potyviruses. This comprises the already known *Celery mosaic virus* and celery latent virus, which has not been intensively described so far and, due to its particle morphology, could belong to the *Potyviridae*. For this virus no sequence data are published.

The objective of the thesis is the molecular and biological description and characterization of CeLV and a new CeMV isolate from Quedlinburg. The complete genome sequence is to be determined and on that basis, a taxonomic classification of both viruses in the phylogenetic tree of the *Potyviridae* (CeLV) or the genus *Potyvirus* (CeMV) is aimed for. In order to fulfil the Koch's postulates and to study protein functions, full-length cDNA clones of both viruses should be constructed using different ligation strategies for example Gibson Assembly followed by *R*. *radiobacter* mediated infection of *A*. *graveolens*.

Information on host plants and symptom development are available from the literature for both species. The establishment of host plant spectra for both viruses is intended to examine, whether these correspond to those described before. With regard to CeMV, there are varying reports on host plant spectra for different isolates. On the one hand there are isolates which are able to infect several plant families and some which are restricted to *Apiaceae* species only. Combined with the determination of the complete genome sequence and comparisons with other CeMV sequences from the NCBI database, an insight into the molecular background concerning putative evolutionary adaptions is expected.

For CeLV, a putative N-terminal localised ER-signal peptide was predicted and its functionality should be proven by co-localization studies. For this purpose, two constructs are planned, which contain on the one hand the viral 5’-UTR plus the following sequence of the signal peptide and on the other hand the 5’-UTR alone as a

negative control. The respective construct is then to be co-infiltrated with an ER-marker labelled with mRFP in order to perform co-localization studies within *N. benthamiana* cells by confocal laser scanning microscopy.

Another hypothesis is that the N-terminus of CeLV contains a silencing suppressor, since the position of such a protein is relatively conserved within the family - it is mostly either the first or the second protein. A transient local silencing assay was planned to answer this question. This assay is based on the triggering of RNA silencing in the plant, which is stopped or slowed down with the help of a potential silencing suppressor. A clone carrying a certain part of the CeLV genome is to be infiltrated in parallel with a construct with the same *gfp* gene as present in a transgenic plant. If no silencing suppressor is active, the fluorescence of the GFP will disappear, but if there is an active suppressor, the fluorescence remains.

These experiments should result in a genome map of CeLV illustrating an overview of the genomic elements and features.

2 Complete genome sequence and construction of an infectious full-length cDNA clone of celery latent virus – an unusual member of a putative new genus within the *Potyviridae*

2.1 Abstract

Celery latent virus (CeLV) has been an incompletely described plant virus known to be sap and seed transmissible and to possess flexuous filamentous particles measuring about 900 nm in length, suggesting it to be a possible member of the ”potyvirus group”. Here, an Italian isolate of CeLV was transmitted by sap to a number of host plants and shown to have a single-stranded and monopartite RNA genome being 11,504 nucleotides in size and possessing some unusual features. The RNA contains a large open reading frame that is flanked by a very short 5’-UTR of 13 nts and a 3’-UTR of 571 nts which is not polyadenylated. The CeLV RNA shared nt sequence identities of only about 40 % with other members of the family *Potyviridae*. The CeLV polyprotein is notable in that it starts with a signal peptide, has a putative *pipo* ORF and shares low aa sequence identities (about 16 %) with other potyvirids. Although the possible cleavage sites were not identified for the N-terminal two thirds of the polyprotein, the latter possesses a number of sequence motifs, the identity and position of which are characteristic of other potyvirids. Attempts at constructing an infectious full-length cDNA clone of CeLV were successful following *R. radiobacter* infiltration of *N. benthamiana* plants. CeLV appears to have the largest genome of all known potyvirids and some unique genome features that may warrant creation of a new genus, for which we propose the name celavirus.

2.2 Introduction

When the umbelliferous crops celeriac (*A. graveolens* var. *rapaceum*) and celery (*A. graveolens* var. *dulce*), are infected by viruses such as *Cucumber mosaic virus* and *Celery mosaic virus*, infected plants typically show mosaic symptoms on the leaves (Severin, Freitag 1938; Severin 1950). In contrast, no visible symptoms are observed

after infection with celery latent virus. CeLV is an incompletely described plant virus that, based upon studies performed about 40-50 years ago, has been known to be sap transmissible to a large number of test plants, to be transmitted at high rates through seeds of celeriac (34 %) and *C*. *quinoa* (67 %) and to possess flexuous filamentous particles measuring about 900 nm in length. Hence, it was considered a possible member of the ”potyvirus group” by M. Hollings & A.A. Brunt (Hollings, Brunt 1981), although all attempts at transmitting it with five aphid species and at finding cytoplasmic cylindrical (“pinwheel”) inclusions in infected tissue failed (Brandes, Luisoni 1966; Bos et al. 1978).

The *Potyviridae* is one of the largest plant virus families and currently contains eight genera harbouring 195 species. However, it has recently been suggested that bellflower veinal mottle virus and rose yellow mosaic virus, currently unassigned members in the family *Potyviridae,* may represent two further genera for which the names bevemovirus and roymovirus*,* respectively, have been proposed (Mollov et al. 2013; Seo et al. 2017b, ICTV-pending proposals). The members of the individual genera are transmitted by different vectors. Viruses of the genera *Macluravirus* and *Potyvirus* are vectored by aphids, those of the genera *Poacevirus, Tritimovirus* and *Rymovirus* by eriophyid mites. Ipomo- and bymoviruses are transmitted by whiteflies and root-infecting organisms of the *Plasmodiophorales*, respectively. The vectors of viruses in the genus *Brambyvirus* and the proposed genera bevemovirus and roymovirus are unknown (Wylie et al. 2017). All monopartite members of the family form flexuous filamentous particles with a length of 680-900 nm and their genome consists of a positive single-stranded RNA ((+)ssRNA) with a poly(A) tail at the 3’-end. Most of the genera have a monopartite genome except the bipartite bymoviruses. Their RNA1 encodes the proteins P3 to CP (coat protein) and RNA2 P1 and P2 and the particle lengths are between 550 and 275 nm respectively (Kashiwazaki et al. 1990; Davidson et al. 1991; Kashiwazaki et al. 1991). In the genus *Potyvirus* the long potyviral open reading frame (ORF) is directly translated into a large polyprotein that is proteolytically processed into ten multifunctional proteins: P1, HC-Pro (helper component-protease), P3, 6K1, CI (cylindrical inclusion), 6K2, NIa-VPg (nuclear inclusion protein a -viral protein genome-linked), NIa-Pro (nuclear inclusion protein a -protease), NIb (nuclear inclusion protein b, RNA-dependent RNA polymerase [RdRp]) and CP (Urcuqui-Inchima et al. 2001). The cleavage into the individual functional proteins is cotranslationally performed in cis by the proteases P1 and HC-Pro and in

trans by NIa-Pro (Carrington, Dougherty 1987; Carrington et al. 1989; Verchot et al. 1991). Besides, the HC-Pro functions as a silencing suppressor in the plant defence mechanism RNAi and is essential for transmission by aphids (Carrington et al. 1989; Blanc et al. 1997; Valli et al. 2017). An additional protein named P3N-PIPO (pretty interesting *Potyviridae* ORF) is encoded by a ribosomal frameshift within the P3 cistron at a conserved $G_{1\text{-}2}A_{6\text{-}7}$ motif. P3N-PIPO is most likely involved in viral short distance movement and could play a role in host-range expansion (Chung et al. 2008; Wei et al. 2010b; Hillung et al. 2013; Olspert et al. 2015).

The number and order of most of the cistrons in the polyprotein is conserved within the family, with highest variation in the N-terminal region. Members of the genera *Poacevirus*, *Potyvirus*, *Rymovirus* and *Tritimovirus* follow the N-terminal pattern of P1, HC-Pro and P3 (Urcuqui-Inchima et al. 2001). The P1 proteins of tritimo- and poaceviruses have been shown to be the silencing suppressor (Tatineni et al. 2012; Young et al. 2012). Blackberry virus Y has the same types of cistrons but its HC-Pro lacks the region which is responsible for the silencing suppressor activity. Indeed, the large P1 includes an AlkB domain being considered to have an alternative role in the silencing suppression (Susaimuthu et al. 2008). The polyprotein of the macluraviruses lacks a P1 and starts with a truncated HC-Pro (Kondo, Fujita 2012; Minutillo et al. 2015; Zhang et al. 2016). Within the genus *Ipomovirus* there are several significant differences. *Sweet potato mild mottle virus* (SPMMV) and *Tomato mild mottle virus* (TMMoV) show the typical potyviral cistrons (Colinet et al. 1998; Abraham et al. 2012) but *Cucumber vein yellowing virus* (CVYV) and *Squash vein yellowing virus* (SqVYV) have a duplicated P1, referred to as P1a and P1b, whereof P1b exerts the silencing suppressor function (Janssen et al. 2005; Valli et al. 2006; Li et al. 2008; Valli et al. 2008; Carbonell et al. 2012). *Cassava brown streak virus* (CBSV) and *Ugandan cassava brown streak virus* (UCBSV) have another type of genome organization by lacking an HC-Pro. In these cases, the P1 functions in RNAi suppression (Mbanzibwa et al. 2009; Monger et al. 2010).

In this study the complete genome sequence of an Italian isolate of CeLV was determined. Furthermore, an infectious full-length cDNA clone was obtained. The results indicate that CeLV has low sequence similarities to other species, and shares only a limited number of amino acid motifs with other potyvirids, suggesting it may represent a member of a putative new genus within the family *Potyviridae*.

2.3 Material and Methods

2.3.1 Origin of virus isolate

The CeLV isolate (Ag097) originated from a plant of *A*. *graveolens* var. *secalinum* from Italy that was received by the Dutch Plant Protection Organisation in 1975. It was propagated in *C*. *quinoa* at IPO in 1990 and 1997. In 2003 Dr. Renè van der Vlugt kindly provided dried plant material to Dr. H. J. Vetten.

2.3.2 Oligonucleotides used for the construction of the infectious full-length clone and verification of CeLV infection

Tab. 2.1: Oligonucleotides used for the construction of the infectious full-length cDNA clone and verification of infection. Underlined parts are vector sequences or restriction sites.

Use of oligonucleotide	Name of oligonucleotide	Sequence of oligonucleotide
Amplification of pDIVA for Gibson Assembly	pDIVA_as	CCTCTCCAAATGAAATGAACTTCCTTATATAG
	pDIVA_s	GGGTCGGCATGGCATCTCCACCTCCTC
Amplification of fragment 1 for Gibson Assembly into pDIVA	CeLV_CEPC_4As	AGGAAGTTCATTTCATTTGGAGAGGAAAATTTAAATTTATGAGCAAG
	CeLV_S3As	GAGATGCCATGCCGACCCGATATTGAATCAGCATGGTAACCAATATCATTC
Amplification of fragment 2 for Gibson Assembly into pDIVA	CeLV456_pCB	AGGAAGTTCATTTCATTTGGAGAGGCCAGCGACTTAAAAGTCAAAGGGT
	CeLV_CEPC_1	GAGATGCCATGCCGACCCTTTTTTTTTTTTTTTTTTTTTTGGAAGTTTTCTGACAG
Amplification of fragment 3 for restriction based ligation into pCASPAR	F5_NotIs	CGTAGCGGCCGCCCATTAATAACCAAGAGCCAATTCC
	CeLV_456neu_AscI	TCTAAGGCGCGCCGGCCGGAACTCCAAAGTAAC
Amplification of CeLV clone for insertion of a smaller part of fragment 3 via Gibson Assembly	CeLV-3end_oeffa	CCTTACGGCAAGGTTCTTAGCTGC
	pDIVA_s	GGGTCGGCATGGCATCTCCACCTCCTC
Amplification of a smaller part of fragment 3 for Gibson Assembly CeLV clone	CeLV_F3endns	GCAGCTAAGAACCTTGCCGTAAGG
	CeLV_3end_pCBas	GAGATGCCATGCCGACCCGGCCGGAACTCCAAAGTAAC
Verification of CeLV infection	CeLV_1s	GGGGGTTGGCACAAACTATACA
	CeLV_1as	TGCTTTCACCCTCTCGGTTAGA

2.3.3 Mechanical inoculation and host range

For the mechanical inoculation, systemically infected plant material was ground in phosphate buffer (0.05 M KH_2PO_4, 0.05 M Na_2HPO_4, 1 mM EDTA, 5 mM Na-DIECA) together with celite and charcoal and rubbed onto two to three mid-aged leaves of a healthy seedling. In order to study the host range of CeLV, three plants of each species were inoculated mechanically with sap from infected *N. benthamiana*. Additionally, one *C. quinoa* plant was inoculated with the virus as a positive control. The verification of an infection with CeLV was done earliest 18 days after inoculation using RT-PCR or back inoculation on *C. quinoa* from locally and systemically infected leaves. The experiments were repeated once. For detailed information about plant species and the supplier of seeds see supplementary table 8.1.

2.3.4 Verification of CeLV infection

To confirm an infection total nucleic acids were extracted using a modified method of Menzel *et al*. (Menzel et al. 2002). For reverse transcription 3 µl of nucleic acid extraction were mixed with 1 µl primer (TGCTTTCACCCTCTCGGTTAGA, 10 µM; salt free; Eurofins Genomics), 4 µl 5X RT Buffer (Thermo Fisher Scientific), 0.5 µl dNTPs (10 mM each; Thermo Fisher Scientific), 1 µl RevertAid Reverse Transcriptase (20 U/µl, Thermo Fisher Scientific) and 10.5 µl H_2O and incubated at 42 °C for 30 min. For the PCR reaction 1 µl cDNA was mixed with 5 µl of FastGene® Taq ReadyMix (2X), 1 µl of each primer (10 µM; salt free; Eurofins Genomics) TGCTTTCACCCTCTCGGTTAGA and GGGGGTTGGCACAAACTATACA and 2 µl H_2O. The PCR program started with 3 min at 95 °C, followed by 28 cycles of 30 s at 95 °C, 30 s at 58 °C and 45 s at 72 °C and a final elongation for 5 min at 72 °C. Samples were analysed on a 1 % agarose gel (w/v), and a wild-type infected *N. benthamiana* as well as a non-infected plant was chosen as positive and negative control.

2.3.5 Sequence determination

For the first sequence determination viral dsRNA was extracted from *N. benthamiana* using a modified method of Morris and Dodds whereupon numerous cDNAs and PCR-fragments were generated, cloned into standard vectors and sequenced (Morris 1979). A combination of virus specific and degenerated primers led to the consensus sequence of a single-stranded positive polarized RNA consisting of 11,220 nucleotides. For determination of the terminal 5'-end, rapid amplification of

cDNA ends was performed (Frohman et al. 1988). For the 3'-end a poly(T) primer was used for cDNA synthesis and combined with a virus-specific primer in PCR.

In a second approach deep sequencing was conducted. For this purpose reverse transcription was carried out in a final volume of 20 µl. First of all 5 µl of purified dsRNA, 1 µl primer GCCGGAGCTCTGCAGAATTCNNNNNN (10 µM; salt free; Eurofins Genomics; originally designed by Froussard (Froussard 1992)), 1.5 µl dNTPs (10 mM each; Thermo Fisher Scientific) and 4 µl H_2O were incubated at 95 °C for 5 min followed by a rapid cooling on ice. Next, 4 µl 5X RT buffer (Thermo Fisher Scientific), 2 µl RevertAid Reverse Transcriptase (25 U/µl; Thermo Fisher Scientific) and 2.5 µl H_2O were added and incubated at 42 °C for 60 min. The resulting cDNA was purified in 20 µl elution buffer with the SureClean Plus system of Bioline. PCRs were carried out using 25 µl Phusion Flash High-Fidelity PCR Master Mix (Thermo Fisher Scientific), 6 µl cDNA, 5 µl primer GCCGGAGCTCTGCAGAATTC (10 mM, salt free, Eurofins Genomics; originally designed by Froussard (Froussard 1992)) and 14 µl H_2O. The amplification started with an initial denaturation at 98 °C for 10 s followed by 35 cycles of 5 s at 98 °C, 5 s at 56 °C, 35 s at 72 °C and a final elongation of 5 min. An aliquot of the reaction was analysed by agarose gel electrophoresis and the rest of the PCR reaction was purified using the NucleoSpin® Gel and PCR Clean-up from Macherey-Nagel following the manufacturer's instructions and an additional precipitation with ethanol and sodium acetate. The deep sequencing reaction was performed by GATC (Germany) on an Illumina HiSeq2500 Genome Sequencer with a 125 bp paired-end HiSeq Rapid Run.

2.3.6 Construction of an infectious full-length cDNA clone

An infectious full-length cDNA clone was constructed using Gibson Assembly where DNA fragments with overlapping domains are assembled in an isothermal enzymatic reaction combined with restriction digest based ligations (Gibson et al. 2009). As the first step, the genome of CeLV was amplified as two fragments of 3,990 bp and 7,287 bp. Both fragments were analysed by gel electrophoresis, purified and introduced into a modified mini binary vector named pDIVA (empty vector map in figure 8.1, KX665539, (Xiang et al. 1999)) via Gibson Assembly separately. The inserts were located downstream of the CaMV 35S promoter followed by a *Hepatitis delta virus* (HDV) ribozyme and a CaMV 35S polyadenylation-signal. Restriction digest analyses identified positive clones and complete sequencing was applied at each step. The

assembly of the complete virus sequence was done via restriction digest based on single sites (*BstE*II and *Bsp*120I). For that purpose the larger fragment was excised and ligated into the digested plasmid containing the smaller fragment. Plasmids were electroporated into *R. radiobacter* and inoculated into *N. benthamiana*. None of the clones was infectious. After deep sequencing and 3'-RACE a 3,807 nt fragment containing the newly found 3'-terminal nucleotides was amplified from dsRNA for insertion into the former non-infectious clones via Gibson Assembly. Because this method was not successful the fragment was ligated into a derivative of pBluescript via restriction digest (*Not*I and *Asc*I). Subsequently a 706 nt fragment from this plasmid was amplified and successfully assembled into the former non-infectious incomplete clones. Plasmids were transformed into *Escherichia coli* NM522 (Hanahan 1983) and after bacterial growth isolated using a modified method of Birnboim *et al*. (Birnboim, Doly 1979). The plasmid map is illustrated in figure 8.2 (left).

2.3.7 Sequence analyses and phylogeny

The deep sequencing data were analysed in Geneious 9 with a "map to reference" against the CeLV sequence after filtering adapter sequences. Phylogenetic analyses were done in MEGA7 and for the alignment using MUSCLE default parameters were chosen (DNA sequences: gap opening: -400, gap extension: 0; protein sequences: gap opening: -2.9, gap extension: 0) (Edgar 2004; Kumar et al. 2016). The phylogenetic tree is based on the complete polyprotein amino acid sequences of 128 members of the family *Potyviridae*, CeLV and a tobacco ringspot virus (*Nepovirus*, TRSV) isolate was used as outgroup. In case of the bipartite species (genera *Bymovirus* and *Nepovirus*), the amino acid sequence of the RNA2 encoded polyprotein was concatenated with the polyprotein of RNA 1. The parameters for the Maximum-Likelihood tree (Le, Gascuel 2008) were set as followed: substitutions type: amino acid, model: LG + frequencies + gamma distributed with invariant sites, test of phylogeny: bootstrap with 1,000 replications, gaps/missing data treatment: complete deletion. For calculation of the percentage identities, sequences of CeLV and the type member of each genus were aligned, respectively. Identity values were displayed in BioEdit 7.2.5 (Hall 1999). A list of all viruses and accession numbers used for tree construction and calculation of the percentage identities is given in the supplementary table. 8.2, 8.3 and 8.4.

2.3.8 *R. radiobacter* GV2260 infiltration of *N. benthamiana*

Putative positive plasmids carrying the complete CeLV genome sequence or the p19 silencing suppressor from tomato bushy stunt virus (TBSV) were transformed into *R. radiobacter* GV2260 via electroporation (Mattanovich et al. 1989; Hellens et al. 2000). Cells and plasmids were mixed and an electric pulse of 1,440 V applied, followed by the addition of 1 ml SOC. After an incubation for 3 h at 28 °C 100 µl were plated on LB with 50 µg/ml kanamycin and incubated at 28 °C for about 36 h. Several colonies were transferred into 15 to 20 ml liquid LB medium with 50 µg/ml kanamycin and incubated at 28 °C for about 24 hours on a shaker (200 rpm). Cells were harvested by centrifugation at 8,000 rpm for 5 min (Eppendorf MiniSpin, F45-12-11) and resuspended in 500 µl inoculation buffer (10 mM $MgCl_2$, 10 mM MES, 100 µM acetosyringone, pH 5.2). The optical densities OD_{600} of all constructs were set to 1.8 ± 0.2 and plasmids containing CeLV and p19 were mixed at a ratio of 3:1. The suspension was infiltrated into the lower surface of several medium aged leaves of four to six weeks old *N. benthamiana* plants using a needleless syringe. Plants were kept in the greenhouse for at least 35 days.

2.3.9 Immuno-electron microscopy

Small pieces (about 5 mm in diameter) of systemically infected leaf tissue were homogenized in 2-5 fold volume of extraction buffer (0.1 M phosphate buffer pH 7.0 containing 2 % polyvinylpyrrolidone MW 11.000 and 0.2 % sodium sulfite). Viral particles were adsorbed by floating a pioloform carbon-coated copper grid on the crude sap preparation. Serological detection using electron microscopy involves immunosorbent electron microscopy (ISEM) and decoration steps which were performed according to protocols by Lesemann (Lesemann 2004). The polyclonal antiserum for serological detection was provided by D. Z. Maat, Wageningen. For negative contrast the grids were stained with 1 % uranyl acetate in ultrapure water, dried and analysed by transmission electron microscopy (TEM) in a Tecnai G2 Spirit electron microscope (FEI Deutschland GmbH, Frankfurt, Germany) using 80 kV accelerating voltage.

2.4 Results

2.4.1 Host range study

The Italian isolate of CeLV was maintained by sap transmission in *C. quinoa* which showed systemic symptoms consisting predominantly of yellow (ring-) spots (Fig. 2.1 (a)). The virus was mechanically transmitted to *N. benthamiana* in which it caused a symptomless systemic infection and sometimes, a slight stunting of the plant could be observed (Fig. 2.1 (b)).

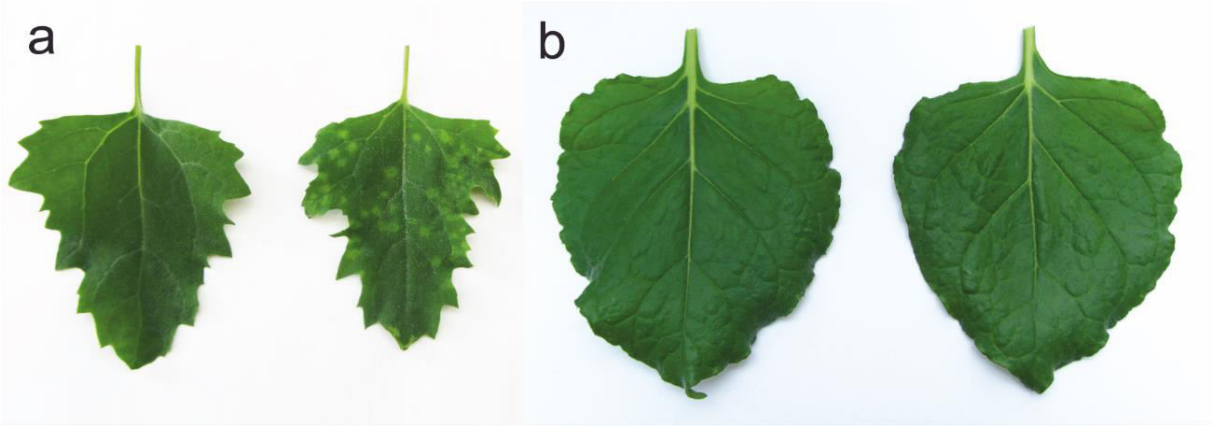

Fig. 2.1: Leaves of *C. quinoa* (a) and *N. benthamiana* (b). Left: non-inoculated; **right:** CeLV wild-type infected.

In host range experiments CeLV was able to infect all celery and celeriac cultivars tested but did not infect three other species of the *Apiaceae* (Tab. 2.2). Different species of the *Amaranthaceae* and several *Nicotiana* species (family *Solanaceae*) were also susceptible to CeLV. In most cases (22 out of 28 species) the infection was latent, only two *Chenopodium* spp. and *Atriplex hortensis* (*Amaranthaceae*) showed local and systemic symptoms. New host plants which have not been reported before are *N. benthamiana* (latent, local and systemic), *N. edwardsonii* (latent, local) and *N. tabacum* var. Samsun (NN & nn: latent, local).

Tab. 2.2: Host range of CeLV.

Plant species	Local infection	Systemic infection	Verification: RT-PCR (RT) or back inoculation on *C. quinoa* (BI)
***Apiaceae/Apium* sp.**			
A. graveolens var. *rapaceum* 'Mars'	I	s	RT
A. graveolens var. *rapaceum* 'Ibis'	I	s	RT
A. graveolens var. *rapaceum* 'Giant Prague'	I	s	RT
A. graveolens var. *rapaceum* 'Monarch'	I	s	RT
A. graveolens var. *rapaceum* 'Sedano rapa'	I	s	RT
A. graveolens var. *rapaceum* 'Prinz'	I	s	RT
A. graveolens var. *dulce* 'Tall Utah 52/70'	I	s	RT
A. graveolens var. *dulce* 'Sedano d'Elne'	I	s	RT
A. graveolens var. *dulce* ‚Bleich-Stangensellerie'	I	s	RT
A. graveolens var. *dulce* ‚Schnittsellerie'	I	s	RT
A. graveolens var. *dulce* ‚Krause Schnittsellerie'	I	s	RT
***Apiaceae*-Other species**			
Coriandrum sativum 'Thüringer'	-	-	BI
Daucus carota L. 'Rote Riesen 2'	-	-	BI
Petroselinum crispum 'Gigante d'Italia'	-	-	BI
Amaranthaceae			
Atriplex hortsensis	L	S	-
Beta vulgaris subsp. vulgaris 'Mangold Lucullus'	I	-	BI
Chenopodium quinoa	L	S	-
Chenopodium amaranticolor	L	S	-
Spinacia oleracea 'Matador'	I	s	RT+BI
Spinacia oleracea 'Monoppa'	I	s	RT+BI
***Solanaceae/Nicotiana* sp.**			
N. benthamiana	I	s	RT+BI
N. clevelandii	I	s	RT
N. edwardsonii	I	-	RT
N. occidentalis	I	s	RT
N. tabacum var. 'Samsun nn'	I	-	BI
N. tabacum var. 'Samsun NN'	I	-	BI
N. tabacum var. 'Xanthi'	I	-	BI
N. tabacum var. 'Xanthi nc'	I	-	BI

L: local symptoms, **S:** systemic symptoms, **I:** latent local infection, **s:** latent systemic infection, **-:** no infection, (same abbreviations as used from Bos *et al*. (Bos et al. 1978)).

2.4.2 Sequence analysis

Based on first sequencing results the genome size was estimated to be 11,220 nts with a poly(A) tail at the 3'-end. Since several clones based on this sequence were not infectious, a deep sequencing approach was used to identify possible sequence variations or putative missing parts of the CeLV genome. The deep sequencing Illumina HiSeq Rapid Run covered nearly the whole CeLV genome. The first 17 nts at the 5'-end were not determined with this method but a stretch of 284 nts starting with the sequence AAAAATACAAAAATATAAAA was identified at the 3'-end. This suggested that mispriming of a poly(T) anchor primer to this internal A-rich region erroneously indicated the presence of a 3'-poly(A) tail in the first experiments. Using different methods for determining viral cDNA ends, the larger 3'-end was verified. However, in several RACE experiments it was not possible to detect a 3'-poly(A) tail. Therefore, the genome of CeLV is a positive-sense single-stranded RNA which consists of 11,504 nucleotides whereof nts 1 to 13 form the 5'-untranslated region (UTR) and nts 10,934 to 11,504 the 3'-UTR (Fig. 2.2). With this genome size CeLV is the largest fully sequenced virus in the family *Potyviridae* to date. The CeLV RNA contains a large ORF of 10,920 nts (starting with an AUG and ending with UAA) that encodes a polyprotein of 3,640 amino acids in length and contains a putative *pipo* ORF. *Pipo* possibly starts at position nts 2,389 with the conserved motif G_2A_7 and terminates with an UGA stop codon at nts 2,962-2,964, resulting in a 190 aa protein with a calculated molecular mass of 22.6 kDa.

Since the polyprotein sequence of CeLV is only distantly related to that of other potyvirids, identification of putative protease cleavage sites in the CeLV polyprotein was difficult. As outlined below it can be assumed that the polyprotein potentially encoded by the CeLV genome is most likely cleaved into at least seven or eight proteins: one or two putative proteins at the N-terminus, P3, CI, NIa-VPg, NIa-Pro, NIb and CP (Fig. 2.2). The CeLV polyprotein shares low similarities with motifs in the CI and NIb proteins of members of the *Potyviridae*, for example to the macluraviruses artichoke latent virus (YP_009129267.1), cardamom mosaic virus (CAC50818.1) and ranunculus latent virus (ADR74229.1) as well as to the bymovirus barley mild mosaic virus (NP_604491.1) and to the potyvirus daphne virus Y (AMR93994.1).

Use of different tools for sequence analyses revealed additional features. DEXDc and helicase domains in the CI as well as RdRp domains of the NIb were identified with SMART (Simple Modular Architecture Research Tool, (Schultz et al. 1998; Letunic et al. 2015) and Blast (Basic Local Alignment Search Tool, (Altschul et al. 1990). Using Phyre2 (Protein Homology/analogY Recognition Engine V 2.0, (Kelley et al. 2015)) NIa and CP structures were predicted. Further sequence analyses revealed a signal peptide at the N-terminus (aa 1-23) right in front of the putative P1 protein. This untypical new genomic element, which potentially leads proteins to the endoplasmic reticulum, is predicted by several tools (Phobius (Käll et al. 2007), iPSORT (Bannai et al. 2002), SignalP 4.1 (Emanuelsson et al. 2007)).

Although the P1 protein is the most variable protein within the *Potyviridae* (Adams et al. 2005a), some conserved motifs were found in the N-terminus of the CeLV polyprotein. The I-X-F-G is available in a slightly altered form V-S-F-G (aa 76-79) and overlaps with a F-G-S-F-T (CeLV: F-G-S-I-T, aa 78-82). A putative C2H2 zinc finger domain C-X_2-C-X_{12}-H-X_{27}-H in the P1 region (aa 265-309) is found. P1 is a serine-protease with the catalytic triad of histidine (H), aspartic acid (D) and serine (S), which is well-conserved in the C-terminal region: H-X_8-(D,E)-$X_{28–31}$-G-X-S (Bazan, Fletterick 1988; Valli et al. 2007; Rohožková, Navrátil 2011). In the CeLV sequence, the amino acids H-224-X_8-D-233-X_{39}-S-273 could form this active site with a larger distance between D and S and/or H-634-X_7-D-642-X_{33}-S-676 indicating the presence of a second or one large serine protease. Serine is typically flanked by a G-X-S-G motif followed downstream by a strictly conserved R-G that is not present in CeLV. The cleavage site should be 22-28 aa downstream of the R-G motif with a consensus sequence of (I, V, L, M)-X-(H, E, Q)-(F, Y)/S. For CeLV this sequence is missing. Two putative cleavage sites are conceivable: I-E-K-Y/V (aa 367-371) or H-V-G-Y/S (aa 700-704). The RNA2 of bymoviruses encodes two proteins, P1 a cysteine-protease and P2 a putative vector transmission factor. For CeLV there is no similar genome organization or a cysteine-like protease detected (Kashiwazaki et al. 1991; Urcuqui-Inchima et al. 2001; Adams et al. 2005a).

Characteristic motifs of HC-Pro proteins (cysteine proteases) like K-I-T-C (aphid transmission), I-G-N (genome amplification), C-C/S-C (long-distance movement) (F,Y)RNK (symptom development), P-T-K (aphid transmission) and G-Y-C-Y (active site of cysteine proteases) are not found in the CeLV polyprotein, indicating that it does

not encode a classical HC-Pro like protein (Oh, Carrington 1989; Atreya, Pirone 1993; Granier et al. 1993; Huet et al. 1994; Cronin et al. 1995; Gal-On 2000; Shiboleth et al. 2007; Mangrauthia et al. 2008; Valli et al. 2017). Possible cleavage sites of a second protein are H-V-G-Y/S (aa 700-704) which could be cleaved by a serine-like protease such as the P1b of CVYV and SqVYV or A-I-V-G/G (aa 735-739) which could be a possible cleavage site for a cysteine-like protease.

There are no similarities in the CeLV sequence corresponding to a P3-like protein apart from the predicted *pipo* ORF, embedded in the P3 cistron. A ribosomal frameshift at a GA_6 motif, highly conserved among members of the *Potyviridae*, results in a fusion protein named P3N-PIPO (Chung et al. 2008; Olspert et al. 2015).

The region comprising the motifs of a CI protein (aa 1202-1856) shows significant similarities to helicase domains of pestiviruses in a BlastP search. In the CI the conserved ‘motif I’ G-X-G-K-S (G-1,274-X-G-K-S-X_3-P-1,282) ‘motif II’ D-E-X-H (D-E-C-H, aa 1,361-1,364), ‘motif III’ T-A-T-P (1,392-1,395) and VI Q-R-X-G-R-X-G-R (aa 1,546-1,553) are present and typical for helicase proteins. Within the CeLV sequence, ‘motifs IV and IVa’ could not be clearly identified whereas the ‘motifs V and Va’ are possibly represented by the amino acids F-A-T-N-A-L-E-S-G-V-T-I (aa 1,498-1,509) (Kadaré, Haenni 1997; Sorel et al. 2014).

Since the CeLV polyprotein lacks significant similarities in the regions corresponding to the 6K1, 6K2 and VPg of other potyvirids, neither cleavage sites nor molecular weights of these putative proteins could be determined. One possible cleavage site between 6K2 and VPg could be L-I-P-E/S (aa 2,296-2,300). The NIa of the potyvirids contains a trypsin-like protease with cysteine instead of serine in the catalytic triad, which cleaves the potyvirus polyprotein at seven sites (Carrington, Dougherty 1987). Its catalytic residues are H, D, C and H whereof the active cysteine is surrounded by the conserved amino acids G-X-C-G. Additionally there is a conserved tryptophan (W) 20 to 36 amino acids downstream of the putative cleavage site (Adams et al. 2005a). These residues can be predicted in the CeLV sequence as H-2,385-X_{31}-D-2,417-X_{72}-G-N-C-G-2,493-X_{13}-H-2,507-X_{39}-W-2,547-X_{15}-I-N-N-Q-2,566, suggesting that CeLV encodes a trypsin-like protease.

The NIb region shows seven of eight conserved motifs known in RdRps. Motif I starts 203 amino acids downstream of the putative cleavage site and contains K-X-E-X_5-K

(aa 2,769-2,777). Additionally, Motif II to VII are detected and contain for example the conserved residues F-T-A-A-P (aa 2,786-2,790, motif II), D-X-S-X-F-D-S-S (aa 2,864-2,853, motif IV), G-N-N-S-G-X_3-T-X_3-N-S (aa 2,903-2,916, motif V) or the highly conserved G-D-D motif (aa 2,951-2,953, motif VI) (Domier et al. 1987; Koonin 1991). A putative cleavage site is predicted to be Q-I-V-Q/S (aa 3,284-3,288) resulting in a large replicase of 721 amino acids.

The downstream CP consists of 353 amino acids and has a calculated molecular mass of 38.64 kDa, which corresponds to results from previous western blots (data not shown). The motifs R-Q (aa 3,407-3,408) and A-F-D-F (aa 3,576-3,579) which were also observed in other potyvirids are present (Sankaralingam et al. 2006; Sudheera et al. 2014). It is known, that the (D, N)-A-G motif in potyviral CPs interacts with the HC-Pro and plays a role in aphid transmission. This motif is not present in the CeLV sequence, which further supports the notion that CeLV is not transmitted by aphids (Atreya et al. 1990; Atreya, Pirone 1993; Atreya et al. 1995). The putative genome organization of CeLV is summarized in figure 2.2 according to the sequence and protein comparisons given above.

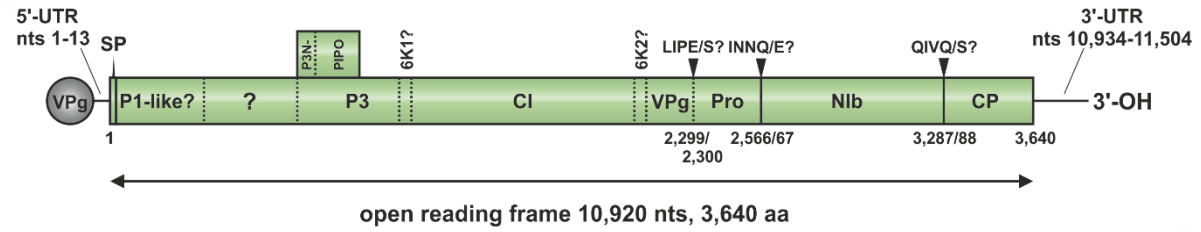

Fig. 2.2: Schematic figure of the putative genome organization of CeLV. Arrows and "?" indicate possible positions of cleavage sites or protein identities. The dotted lines indicate possible beginnings and ends of the corresponding element. **UTR:** untranslated region; **nts:** nucleotides; **aa:** amino acids

2.4.3 Phylogeny and taxonomic classification

In a phylogenetic tree (Fig. 2.3) the CeLV sequence was compared with representatives of all established and proposed genera in the *Potyviridae*, using tobacco ringspot virus as an outgroup sequence. Since CeLV does not cluster to any of the known genera so far, it forms a distinct branch being positioned close to the *Bymovirus* and *Macluravirus* branches, indicating the possible existence of a new genus.

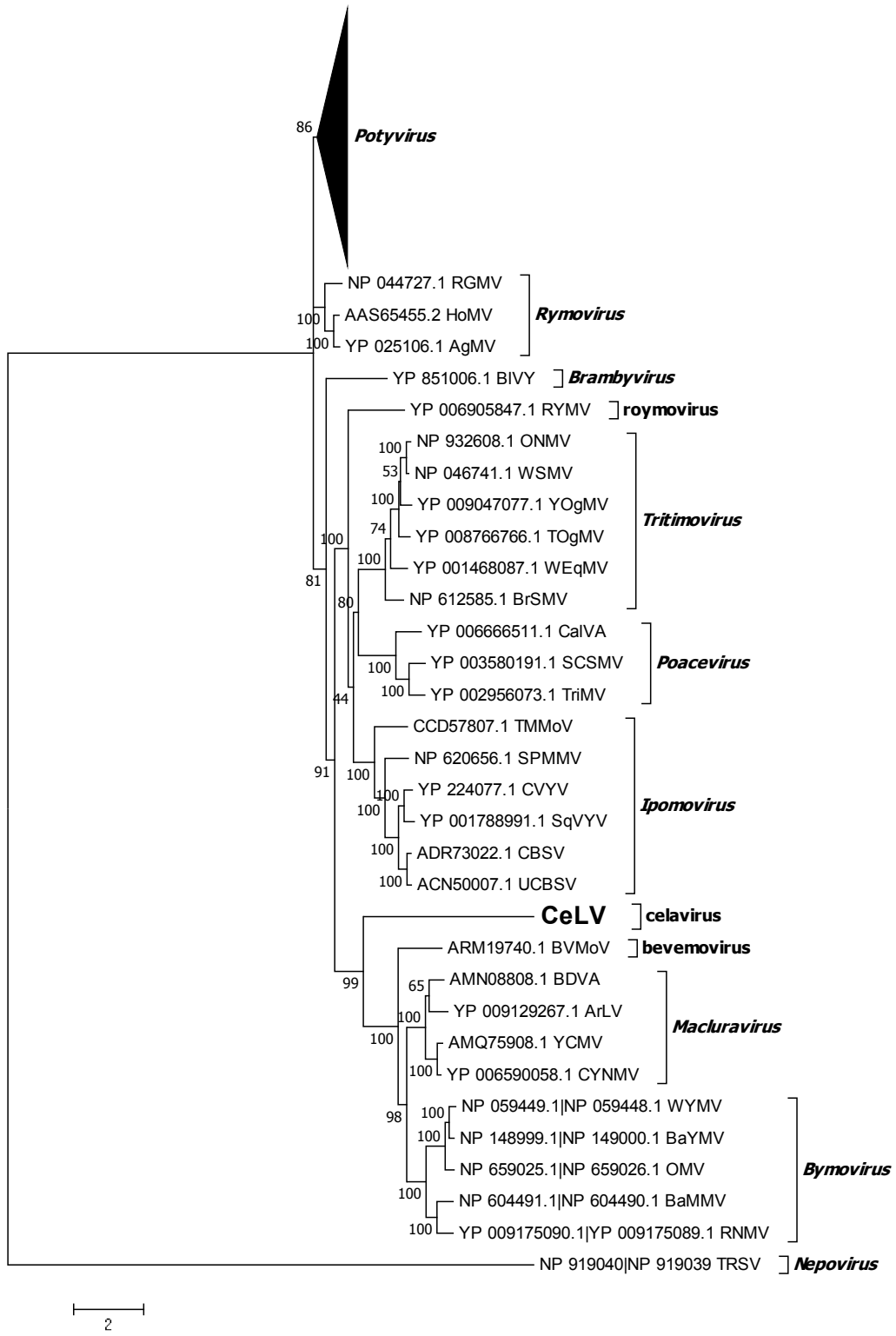

Fig. 2.3: Phylogenetic tree based on 128 complete polyprotein sequences of viruses in the family *Potyviridae*, celery latent virus and tobacco ringspot virus (TRSV, *Nepovirus*) as outgroup. The genus *Potyvirus* consists of 98 sequences (black filled triangle, accession numbers are listed in table 8.2). Alignment was performed by the Muscle alignment tool (Gap open: -2,9, gap extension: 0) (Edgar 2004) and tree construction was done using the Maximum-likelihood method (model: LG + frequencies + gamma distributed with invariant sites; bootstrap: 1,000 replicates) in MEGA7 (Kumar et al. 2016). The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. All positions containing gaps and missing data were eliminated.

In addition, the percent identities of the nucleotide and amino acid sequences of the polyprotein, NIb and CP of each type member in comparison to CeLV were calculated (Tab. 2.3).

Tab. 2.3: Percent identities in nucleotide and amino acid sequences of the polyprotein, nuclear inclusion protein b (NIb) and capsid protein (CP) between CeLV and the type members of the individual genera of the *Potyviridae*. Highest values are indicated in bold.

	entire ORF/polyprotein		NIb		coat protein	
	nts	aa	nts	aa	nts	aa
Bevemovirus BVMoV	37.7	16.5	39.4	23.0	39.8	15.3
Brambyvirus BlVY	**42.6**	16.7	41.8	**23.2**	40.9	15.9
Bymovirus BaYMV	41.7	16.0	39.3	22.4	41.6	14.7
Ipomovirus SPMMV	**42.6**	15.6	**42.3**	21.8	39.7	13.9
Macluravirus CYNMV	40.1	16.4	41.7	22.3	**43.4**	**18.4**
Poacevirus TriMV	41.8	16.4	37.6	19.4	39.6	16.0
Potyvirus PVY	41.4	16.3	40.2	21.2	37.5	13.7
Roymovirus RYMV	41.9	**17.4**	39.8	20.9	41.2	15.3
Rymovirus RGMV	42.3	16.6	40.2	21.1	42.7	13.8
Tritimovirus WSMV	42.0	16.0	39.9	19.8	42.3	16.1

Comparisons of the nucleotide and amino acid sequences of the large open reading frame and polyprotein sequences range from 37,7 % (*Macluravirus*, CYNMV) to 42.6 % (*Brambyvirus*, BlVY and *Ipomovirus*, SPMMV) and from 15.6 % (*Ipomovirus*, SPMMV) to 17.4 % (*Roymovirus*, RYMV), respectively. In the NIb, CeLV and other potyvirids share nucleotide and amino acid sequence identities ranging from 37.6 % (*Poacevirus*, TriMV) to 42.3 % (*Ipmovirus*, SPMMV) and from 19.4 % (*Poacevirus*, TriMV) to 23.2 % (*Brambyvirus*, BlVY), respectively. For the CP sequences, the respective values range from 37.5 % (*Potyvirus*, PVY) to 43.4 % (*Macluravirus*, CYNMV) and from 13.7 % (*Potyvirus*, PVY) to 18.4 % (*Macluravirus*, CYNMV).

2.4.4 Construction of an infectious full-length cDNA clone and infiltration of *N. benthamiana*

N. benthamiana plants that had been infiltrated with *R. radiobacter* cultures were kept in the greenhouse for 35 to 50 days and observed for symptom development after infiltration. As shown for the wild type virus before, no symptoms developed during these periods. In five experiments using ten infiltrated plants each, the infection rates were relatively low ranging from one to four infected plants. Sequencing of selected positions of the viral RNA isolated from the infected plants confirmed single nucleotide polymorphisms and amino acid exchanges, which only occured in the full-length cDNA clone and were not found in the deep sequencing approach of the wild-type virus, corroborating the infectivity of the full-length clone. In addition, electron microscopy of crude sap preparations from infected plants following infiltration with a CeLV full-length cDNA clone, revealed the presence of flexuous filamentous particles. The latter were decorated when an antiserum produced by D. Z. Maat (Wageningen, The Netherlands) in 1978 was used in immunoelectron microscopy (Fig. 2.4).

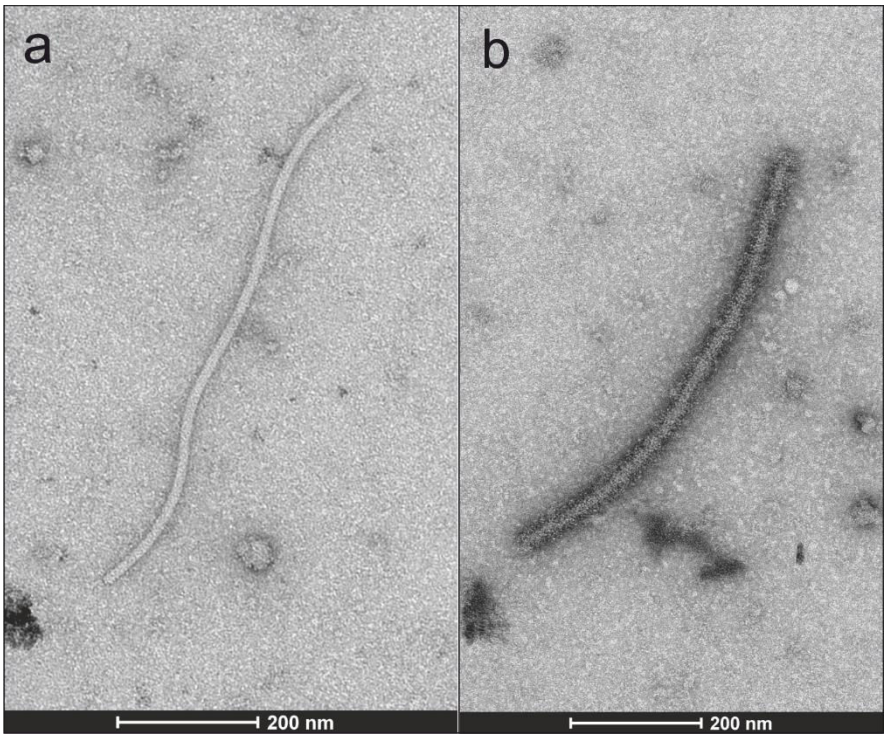

Fig. 2.4: **Electron micrograph of a CeLV particle visualized from an infected *N. benthamiana* plant following infiltration with a full-length cDNA clone of CeLV**. **a:** CeLV particle undecorated **b:** particle decorated with CeLV-specific antibody.

Attempts to infect *C. quinoa* and *A. graveolens* with the full-length clone via *R. radiobacter* infiltration were not successful. To test if the virus derived from the

plasmid is able to infect these plants, a transmission experiment was carried out using sap from a full-length clone infected *N. benthamiana* plant for mechanical inoculation of three plants each of *N. benthamiana*, *C. quinoa* and *A. graveolens*. In three replications, all inoculated *N. benthamiana* and *A. graveolens* plants have been tested positive but no infection was detected in *C. quinoa*. The sequencing of the clone revealed that there were six mutations compared to the contig from the deep sequencing, whereof three led to an amino acid exchange V16I (putative first protein), N2392D (NIa) and V3158I (NIb) (wild-type/clone).

2.5 Discussion

Initial studies of CeLV and its host plant spectrum were carried out in 1966,1976 and 1978 and showed slightly varying results when compared to each other (Bos et al. 1978). As expected, the Italian CeLV isolate Ag097 used in this work was able to infect plants from the families *Solanaceae*, *Amaranthaceae* and *Apiaceae*. Compared to the summarized results of Bos et al., only one difference in the case of coriander has been detected, which could not be confirmed as a host plant in the present work possibly resulting from the use of different varieties. More potential hosts from the *Apiaceae*, such as fennel (*Foeniculum vulgare*), chervil (*Anthriscus cerefolium*) and lovage (*Levisticum officinale*), would have to be tested to answer the question whether CeLV can infect more species of this family than *Apium*.

CeLV is currently the largest of all sequenced members of *Potyviridae* and shows high sequence deviations to other viruses, with the consequence that it cannot be assigned to any of the known and proposed genera. This fact is supported by the phylogenetic analysis (Fig. 2.3) in which CeLV was positioned near to the *Bymovirus* and *Macluravirus* branches but with a distance indicating the formation of a new genus. This is further elucidated by the calculated percentage identities (Tab. 2.3), which are below the established values for genus demarcation. For the polyprotein, the values should be below 46 % on nucleotide level and 33 % on amino acid level and for the NIb and CP regions below 54 % and 49 % on nucleotide level (Adams et al. 2005b). Derived from the species name, celery latent virus is proposed as the type member of a new genus with the designation celavirus. However, there are special features that contrast with this classification. There is the lack of a poly(A) tail at the 3'-end and of

cylindrical inclusion bodies, which are regarded as a typical characteristic of potyviruses (Bos et al. 1978; Sorel et al. 2014). In addition, a predicted signal peptide is a unique new element. The potyviral CI is part of the superfamily 2 helicases and reported to be related to homologous proteins of flaviviruses and pestiviruses (Laín et al. 1989). In a BlastP analysis, the putative CI region of CeLV (aa 1202-1856) shows more similarities to *Norway rat pestivirus* (NrPV) and *Bovine viral diarrhea virus* (BVDV1) than to plant viruses or even potyviruses. It could be assumed that this characteristic implements a sequence-based or structural difference which leads to reduced CI self-interaction and as a consequence no formation of pinwheel inclusions. Detailed analyses of the genome and polyprotein sequence with different algorithms and tools revealed only weak similarities to known coding sequences, amino acid motifs or proteins of other viruses. But the identity and position of the motifs which could be found are characteristic for members in the family *Potyviridae* supporting the classification (Urcuqui-Inchima et al. 2001; Wylie et al. 2017). With reference to literature and current knowledge, it was not possible to determine all exact protease cleavage sites due to the large sequence deviations apart from those between NIa-pro and NIb as well as NIb and CP (Adams et al. 2005a). Sequence analysis of the N-terminal part of the polyprotein revealed a putative zinc finger domain. C2H2 zinc finger domains within the P1 N-terminus have been reported for some potyviruses and two tritimoviruses but the function remains unknown (Valli et al. 2007; Rohozková et al. 2014). The results do not allow an exact determination of the protein identities in this area. There are indications of two putative serine proteases, but the motifs and potential cleavage sites deviate from the conserved patterns. It would be necessary to investigated whether the proteins in question function autocatalytically or if they might be cleaved by the NIa, too. The functions of P1 are still not well understood. Until now it is known to be involved in silencing suppression, enhances amplification and movement of a virus, has effects on the host range but is not strictly essential for infection. Localization studies revealed that P1 of TEV can also be found trafficking into and out of the nucleus and binding to the hosts 60S ribosomal subunits during infection (Martinez, Daros 2014). If CeLV contains a P1-like protein a protease function is not evident from the sequence. In consequence, it is not clear if the putative P1 after proteolytic release or P1 connected to the polyprotein is guided by the signal peptide to or into the ER. It is likely, that CeLV does not encode a cysteine-protease or HC-Pro like protein because all corresponding known motifs are missing (Valli et al. 2017). The

lack of vector transmission-associated motifs may be supported by the observation that attempts at using five different aphid species for CeLV transmission failed (Bos et al. 1978). Thus, CeLV does not appear to be transmitted by aphids but predominantly through seeds in its hosts celery and celeriac. It remains to be shown whether under natural conditions CeLV has another type of vector or is transmitted via seeds in its hosts celery and celeriac. Therefore, the function of the putative second protein remains unclear, like processing of the N- and C-terminal parts of this portion of the polyprotein. Although less is known about the first proteins, a silencing suppressor is likely located in this part. Plasmids containing different N-terminal parts of the CeLV genome could be used to address the question if CeLV has a silencing suppressor as shown for CVYV, CBSV and PPV by using a transient local silencing assay in *gfp* transgenic *N. benthamiana* 16c plants (Valli et al. 2006; Varrelmann et al. 2007; Mbanzibwa et al. 2009).

The construction of an infectious full-length cDNA clone was successful and an infection could be verified at least 35 dpi. The infection rates in several repetitions were very low and differed between one and four out of ten inoculated plants. To improve the infection rate with the full-length CeLV clone other *R. radiobacter* strains and/or different optical densities of the bacteria in the inoculation buffer can be used (Hellens et al. 2000). Additionally, another binary vector system like pCAMBIA with a pVS1 replicon could be used to investigate and improve the stability of the clone in *R. radiobacter* (Murai 2013). Low infection rates could be also attributed to plant-based cellular mechanisms during *R. radiobacter* transformation, in which a DNA segment (here the full-length CeLV sequence together with a promoter and termination signals) is transported into the cell nucleus (Grimsley et al. 1986; Gelvin 2003). There, pre-mRNA is transcribed, processed and transported out of the nucleus into the cytoplasm for translation. Potyviruses replicate in the cytoplasm and do not have a replication stage in the nucleus (Mäkinen, Hafrén 2014). In case of agroinfection full-length transcripts of clone-derived mRNA might be inefficiently produced, degraded by splicing mechanisms or not exported from the nucleus. To circumvent some of these problems, insertion of one or multiple introns with stop-codons inside, for example into the P3 or CI regions, could improve the stability and amplification of full-length clones in *E. coli* or *R.* radiobacter. Viral RNA is then re-established in the plant after mechanical or biolistic treatment (Johansen 1996; Lopez-Moya, Garcia 2000; Tuo et al. 2015). Furthermore, the removal of multiple putative cryptic splice sites and

insertion of 16 introns into a TMV-based expression vector resulted in higher transfection rates, increased viral replication and higher protein yield (Marillonnet et al. 2005). For CeLV putative cryptic splice sites were predicted by the NetGene2 World Wide Web Server (Hebsgaard et al. 1996). Therefore, a possible approach to increase the infection rate of CeLV is also an insertion of silent mutations into these positions to prevent splicing events in the nucleus. The fact that the clone in *Rhizobium*-infected plants could infect celery and tobacco, with an infection rate of 100 %, after mechanical transmission, indicate that the low infection rates and non-infectivity in celery, as observed after *R. radiobacter* infiltration, are not caused by mutations within the clone but probably by the inoculation method. The loss of infectivity in *C. quinoa* might be explained by the six mutations in the nucleotide sequence (compared to the contig from deep sequencing), which results in three amino acid changes V16I, N2392D and V3158I (wild-type/clone). Especially amino acid position 16 could be significant, as it is located in the putative signal peptide/P1, which is known to influence the host-range (Salvador et al. 2008b). The other mutations are in the putative NIa and NIb regions. To investigate if one or a combination of these mutations cause the failure to infect *C. quinoa*, mutations back to the wild-type sequence are suitable. The infectious full-length cDNA clone is of great importance for investigating the biology of CeLV. For example, it is now possible to generate C-terminally shortened versions for coupled *in vitro* transcription and translation systems to investigate proteolytic activity for estimation of the size and number of proteins located at the N-terminal region of the polyprotein. The suitability of this approach was successfully applied for other viruses like TEV (Verchot et al. 1991). To determine the exact protein cleavage sites, protein sequencing will be necessary.

Although more information on the biology of CeLV is needed, it appears to be an unusual new member of a new putative genus celavirus in the family *Potyviridae*.

3 Investigations on the functionality of the putative signal peptide and identification of a suppressor of gene silencing in the genome of celery latent virus

3.1 Abstract

CeLV has been shown to be an unusual member of a putative new genus celavirus in the family *Potyviridae* with a N-terminal localised signal peptide as the only predicted feature within the first third of the genome. This element is so far unique for potyvirids and in this study the functionality could be verified. In co-localization studies using plasmids containing the 5'-UTR with or without the signal peptide fused to *gfp* and an mRFP coupled ER-marker, the signal peptide was shown to lead proteins to or into the endoplasmic reticulum. Further experiments targeted the localisation and identification of a putative silencing suppressor protein to get a first insight to the identity of N-terminal located proteins. For this, a transient local silencing assay was performed using *gfp* transgenic *N. benthamiana* 16c. Clones were constructed comprising parts of the CeLV genome with conserved motifs like DECH in the CI or putative cleavage sites and compared to equally designed PPV clones. With this method it was possible to verify a silencing suppressor activity in the N-terminal part of the CeLV polyprotein and to narrow down the region to the amino acid positions 1 to 703. Different N-terminal compositions and polyprotein features of CeLV are discussed.

3.2 Introduction

Within a plant cell multiple organelles are present and numerous proteins have to be sorted to or into them depending on their final task. This sorting is regulated by signals which have to be recognized, for example signal peptides recognized by the signal recognition particle (SRP) receptor in the endoplasmic reticulum membrane. In this case, an N-terminal signal peptide of a polyprotein chain is bound by an SRP, which locates near to the ribosome and stops the translation. This complex interacts with the SRP receptor in the ER membrane and the SRP is released so that translation can be continued. Opening a pore enables the protein import into the ER lumen where the

signal peptide is cleaved off the chain (reviewed in (Walter, Johnson 1994; Paetzel et al. 2002)). Plant viruses use the ER mostly for movement or replication. It was shown that the TMV MP associates with the ER/actin-network to reach the plasmodesmata for short-distance movement (Sambade et al. 2008). *Brome mosaic virus* (BMV, *Bromovirus*) expresses the two replication proteins 1a and 2a being part of the replication complex, which both co-localize with the ER (Restrepo-Hartwig, Ahlquist 1996; Chen, Ahlquist 2000). Potyviruses use their 6K2 protein as an ER membrane anchor for the replication complex (Wei et al. 2010a). All these features do not include N-terminal localised signal peptides as described above. We assume that CeLV is the first member of the *Potyviridae* with a signal peptide being able to lead proteins to the ER.

All members within the *Potyviridae* encode a silencing suppressor in the N-terminus to overcome the plant defence mechanism. The first RNA silencing/RNA interference (RNAi) effects were observed in 1990 in experiments intending to achieve a higher pigmentation of petunia petals by overexpression of a chalcone synthase (Napoli et al. 1990). Contrary to the expectations, a high amount of plants showed white or mostly whitened petals, indicating an inhibition of the synthesis, as well as a reduction of the corresponding mRNA level. It could be shown that the transcription is not inhibited but the product (mRNA) is degraded (van Blokland et al. 1994). A similar mechanism was observed in *Caenorhabditis elegans* by introducing antisense constructs targeting myofilament genes that resulted in reduced transcript and protein levels (Fire et al. 1991). The RNAi was studied intensively showing to be a sequence specific mechanism to degrade RNA and to date the general process is well researched. The triggering dsRNA molecules are recognized by an RNase III-like enzyme complex (DICER) and digested into short interfering RNAs (siRNAs) with a size of 21 to 25 nucleotides being homologous to the corresponding gene (Hamilton 1999; Bernstein et al. 2001). The siRNAs associate with the RNA-induced silencing complex (RISC) and guide it to the complementary mRNA or viral RNA. The degradation of the targeted molecules is the consequence (Mlotshwa et al. 2002). Plants have evolved a similar mechanism called post-transcriptional gene silencing (PTGS) as a defence mechanism against for example transgenes and foreign nucleic acids, like viruses. The virus-induced gene silencing (VIGS) is triggered by dsRNA, an intermediate product which occurs during viral replication, which leads to degeneration of the dsRNA and homologous single-stranded RNA (ssRNA) (Voinnet 2001). PTGS effects were

observed to spread throughout the plant and are not restricted to the initiation spots (Boerjan 1994; Voinnet, Baulcombe 1997). This feature led to recovered plants being resistant to a new infection with the virus or closely related ones (Covey et al. 1997; Ratcliff 1997). To overcome the plant defence, viruses have evolved various silencing suppressors that interrupt the PTGS at different stages. To date numerous silencing suppressors of viruses have been found showing different structures, strategies and sequences (Li, Ding 2006). In the genus *Tombusvirus* (*Tombusviridae*) with the type member *Tomato bushy stunt virus* the silencing suppressor is the p19 protein. It was shown that this protein can inhibit the formation of the RISC complex by sequence-independent binding of siRNAs plus inhibiting their systemic spread (Silhavy et al. 2002; Qu et al. 2003; Ye et al. 2003; Lakatos et al. 2004). Another strategy is pursued by CMV (*Cucumovirus*, *Bromoviridae*) which expresses the 2b protein to bind the double-stranded siRNAs on the one hand and Argonaute1-proteins (AGO, part of the RISC) on the other hand (Guo, Ding 2002; Zhang et al. 2006). Recently, the independency of the 2b-AGO binding ability and the RNA binding was shown, demonstrating that siRNA binding was essential for induction of virulence and 2b-AGO interaction for interference of RNA-dependent RNA polymerase mediated silencing (Duan et al. 2012; Fang et al. 2016). Within the *Potyviridae*, the most occurring silencing suppressor is the HC-Pro located in the N-terminal part (mostly second protein) of the polyprotein (Anandalakshmi et al. 1998). This protein interferes with the silencing mechanism by impeding the siRNA accumulation but it is not able to stop the mobile spread of the signal (Mallory et al. 2001; Lakatos et al. 2006). A distinct conserved motif, the F-R-N-K box, is considered to be responsible for the interaction with the siRNAs (Shiboleth et al. 2007). Recent studies identified host components responsible for methylation as HC-Pro interaction partners resulting in inhibition of the methylation and destabilization of small RNAs as well as a putative impact of a “ribosome-associated, virus-specific complex comprising HC-Pro, CI and VPg/VPg-Pro” on AGO1 (Ivanov et al. 2016). This illustrates the complex molecular processes in viral silencing suppression and that detailed functions still have to be investigated. The HC-Pro is not the only silencing suppressor within this family. Blackberry virus Y HC-Pro lacks the N-terminal part which is responsible for suppressor activity being replaced by an AlkB domain in the C-terminal part of a large P1 protein considered to be an alternative silencing suppressor (Susaimuthu et al. 2008). Within the ipomoviruses, CVYV and SqVYV have a duplicated P1 in form of

P1a and P1b with the latter as silencing suppressor, whereas CBSV and UCBSV have a P1 and no HC-pro with P1 exerting the silencing function (Valli et al. 2006; Mbanzibwa et al. 2009; Monger et al. 2010). It could recently been observed that the SPMMV P1 protein is responsible for the suppression of silencing (Kenesi et al. 2017). This variant could also be demonstrated in the poaceviruses and tritimoviruses (Tatineni et al. 2012; Young et al. 2012). Taken together silencing suppression of potyvirids has no consistent strategy or protein identity but all variants have one common feature: the suppressor proteins are located within the N-terminal part of their respective polyproteins indicating a conservation regarding the position.

In the CeLV polyprotein there is a very short sequence stretch matching to P1 of several potyviruses but no hint to the existence of a HC-Pro like protein. The identification of a silencing suppressor by sequence or homology modelling analyses was not possible but, due to the taxonomic relatedness to potyvirids, the location of a suppressor in the N-terminal part of the polyprotein is likely. In this work, the demonstration of a silencing suppressor activity in the N-terminal part of the CeLV polyprotein by using a transient local infiltration assay in *gfp* transgenic *N. benthamiana* 16c was successful. However, the exact protein sizes with putative cleavage sites has still to be confirmed.

3.3 Material and Methods

3.3.1 Construction of plasmids for co-localisation studies

To investigate the functionality of the predicted signal peptide, two plasmids, CeLV ΔSP-GFP and CeLV +SP-GFP were constructed in the pDIVA backbone (Fig. 8.1, KX665539) with a nosT replacing the HDV ribozyme and the CaMV 35S polyadenylation-signal. The GFP is a soluble-modified red-shifted (smRS-GFP) variant. CeLV ΔSP-GFP contains 13 nts representing the viral 5'-UTR followed by the *gfp* gene and CeLV +SP-GFP owns the first 88 nts comprising the 5'-UTR and coding sequence for the first 25 amino acids.

3.3.2 Co-localisation experiments and confocal laser scanning microscopy

Plasmids containing an mRFP-ER-marker, CeLV 5'-UTR ΔSP-GFP or CeLV with 5'-UTR+SP-GFP were transformed into *R. radiobacter* GV2260 and treated as

described before. The optical densities OD_{600} of all constructs were set to 1.0 ± 0.1. For inoculation into *N. benthamiana* the ER-marker-mRFP was mixed with either the CeLV 5'-UTR ΔSP-GFP or CeLV 5'-UTR +SP-GFP in a ratio 1:1. Three to four days after inoculation samples were analysed with a confocal laser scanning microscope. Samples were excised from infiltrated leaf-areas and placed under a Leica TCS SP2 microscope. Filters for mRFP detection were set to 543 nm for excitation and 600-610 nm for emission, for GFP detection wavelengths were 488 nm and 510-515 nm, respectively.

3.3.3 *N. benthamiana* 16c and plasmids pBI 16cgfp and pBI HC-Pro

The transgenic *N. benthamiana* line 16c carries an *mGFP5* gene (homozygous) expressing GFP within the complete plant (Haseloff et al. 1997; Ruiz 1998). Recent studies revealed that the GFP variant is an *mGFP5-ER* (containing ER targeting and ER retention signal) with a C-T mutation resulting in a H46T amino acid exchange. Additionally, a transposon co-integrated into the plant genome directly following the *gfp* (Philips et al. 2017). The plants were kindly provided by David Baulcombe. The plasmid pBI 16cgfp carries the identical *gfp* variant described before but lacks the ER targeting and ER retention signal. The construct was integrated in a pBIN vector backbone flanked by a CaMV 35S promotor and nosT terminator. The pBI HC-Pro was constructed by Mark Varrelmann.

3.3.4 Construction of plasmids for the local silencing assay

In order to study silencing suppression effects of CeLV and PPV (pCB:p35PPV_NAT; positive control), first partial clones of full-length cDNA clones were constructed by successive shortening the 3'-part of the genome. To compare the results, conserved sequence motifs or cleavage sites existing in both of the genomes were chosen as for example the DEXD domain in the CI, the GDD motif in the NIb or the putative cleavage site of a HC-Pro like protein (AIVG/G in CeLV). The clones are named with the virus species CeLV or PPV first, followed by the first and/or last encoding aminoacids. As an example: CeLV DECH contains the viral genome sequence ending with the nucleotides encoding DECH in the CI. The 5' shortened clones harbour remaining amino acids from the P1 C-terminus at the N-terminus of the potyviral HC-Pro or putative second protein of CeLV. Effects of 70 remaining amino acids for PPV (FANT clones) and 65 amino acids for CeLV (DPPH clones) as well as 13 amino acids (PPV KVSK and CeLV SIAT clones) should be investigated.

To the clones with a shortened 3'-region, an UAA stop codon for CeLV and UAG for PPV was added behind the viral sequence directly followed by the HDV ribozyme of the vector backbone, thus removing the 3'-UTR. The PCRs were carried out using two adjacent oligonucleotides, a universal sense oligonucleotide which comprises the respective stop codon of the viruses at its 5'-end and a specific antisense oligonucleotide located in the viral sequence. With this method, the sequence stretches between the oligonucleotides are deleted. For the PCR reactions, standard assays were applied using Phusion Flash High-Fidelity PCR Master Mix (Thermo Fisher Scientific). Amplification products were incubated with *Dpn*I (20 U/µl; Thermo Fisher Scientific) for 60 minutes at 37 °C to degrade the original template. Digested products were purified in 20 µl elution buffer with the SureClean Plus system of Bioline. Phosphorylation was carried out in a final volume of 20 µl using 7 µl purified *Dpn*I treated PCR product, 2 µl T4 DNA Ligase Buffer (10X; Thermo Fisher Scientific), 1 µl ATP (10 mM; Thermo Fisher Scientific), 10 U/µl T4 Polynucleotide Kinase (Thermo Fisher Scientific) and 9 µl H_2O and incubated at 37 °C for 60 minutes. Some constructs were first cloned without a stop codon which was introduced afterwards to guarantee the same composition and comparability of all clones. The stop codon was introduced using overlapping oligonucleotides containing the respective nucleotides. For the ligation the Gibson Assembly method was performed. This method was applied for the clones CeLV GDD, DECH, AIVG, IEKY and PPV GDD, DECH, P1+HC-Pro and P1. For the clones CeLV DECH-GDD, PPV DECH-GDD and PPV HC-Pro the inserts were amplified completely and ligated into the empty pDIVA via Gibson Assembly. Leader sequences and start codons (CeLV and PPV DECH-GDD, PPV HC Pro) were introduced afterwards as described in the next step.

The clones which were shortened from the 5'-region were constructed using the Gibson Assembly method (Gibson et al. 2009). Additionally, the 5'-UTR was replaced by insertion of a so called leader sequence (32 nucleotides; ggatccaagcttatcgatt<u>aggagatataaca</u>) comprising an optimized ribosome binding site and optimal context for the start codon (underlined). This element was introduced analogous to the previously constructed pBI HC-Pro (PPV HC-Pro). For the modified Gibson Assembly method, a universal antisense primer was designed, carrying 16 nucleotides overlap to the vector and the first 24 nucleotides of the leader. The corresponding specific sense primer comprises the last 18 nucleotides of the leader, resulting in ten centred overlapping nucleotides to the antisense primer, a start codon

and around 19 nucleotides of the virus sequence. As described before, PCRs were carried out in standard assays and the region between the olinucleotides is deleted. This method is a religation via Gibson Assembly of overlapping regions at the 5'- and 3'-end within one fragment. The following procedure (without phosphorylation) was performed as described before. All fragments were transformed into *E. coli* NM522 and resulting plasmids analysed by restriction digest and/or sequencing (Seqlab Microsynth, Göttingen, Germany) (Hanahan 1983; Birnboim, Doly 1979). To the constructs PPV FANT-DECH and FANT-YLVG the leader sequence and start codon were added using adjacent primers with following phosphorylation. For detailed information about oligonucleotides see table 8.5 in the addendum. In figure 3.1 a scheme illustrates the two different construct compositions.

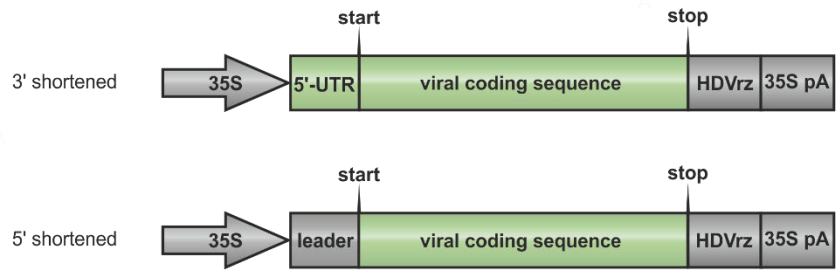

Fig. 3.1: Schematic illustration of the composition of the silencing constructs. The 3'-shortened constructs own the viral 5'-UTR, the 5'-shortened clones have a leader sequence in front of the viral coding sequence**. 35S:** CaMV 35S promoter, **5'-UTR:** 5'-untranslated region of CeLV or PPV; **HDVrz:** *Hepatitis delta virus* self-cleaving antigenomic "core" ribozyme; **35S pA:** polyadenylation signal of CaMV.

3.3.5 *R. radiobacter* GV2260 infiltration of transgenic *N. benthamiana* 16c

Plasmids containing the CeLV or PPV silencing constructs or pBI 16cgfp were treated as described in chapter 2.3.8. Right before infiltration the bacterial suspensions of the respective silencing construct and pBI 16cgfp were mixed in a ratio 1:1.

Plants were kept in the greenhouse and *gfp* expression documented at 3, 5 and 7-9 dpi, unless otherwise stated by visual illumination with a hand-held long-wave UV lamp (Blak Ray model B100 AP). The co-infiltrated GFP triggers the PTGS reaction of the plant resulting in a loss of fluorescence whereas in the presence of a silencing suppressor the signal should remain over time.

3.4 Results

3.4.1 Co-localisation studies to verify the putative signal peptide

The putative signal peptide is predicted to be 23 amino acids in length and has been identified by several algorithms like Phobius (Käll et al. 2007), iPSORT (Bannai et al. 2002) and SignalP 4.1 (Emanuelsson et al. 2007). Phobius assigns the N-region to amino acids 1-6, H-region to 7-18 and the C-region to 19-23.

To study its functionality, the constructs CeLV without and with the signal peptide (SP) fused to GFP were constructed (Fig. 3.2).

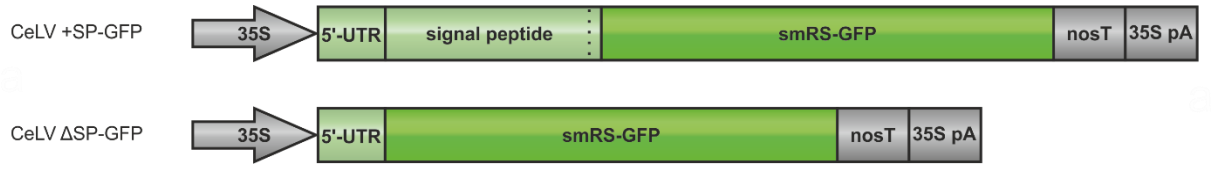

Fig. 3.2: Schematic illustration of the CeLV co-localisation constructs with and without signal peptide. 35S: CaMV 35S promoter, **5'-UTR:** 5'-untranslated region of CeLV (nucleotides 1-13); **signal peptide:** nucleotides 14 to 82 (aa 1-23); **dotted line and area behind:** putative cleavage of the signal peptide and six additional nucleotides (two additional amino acids); **smRS-GFP:** soluble-modified red-shifted GFP; **nosT:** nos terminator; **35S pA:** polyadenylation signal of CaMV.

The co-localisation experiments (Fig. 3.3) reveal different fluorescence patterns of the ER-marker-mRFP (Fig. 3.3 (1a+ 2a) compared to the CeLV constructs without (Fig. 3.3 (1b)) and with the signal peptide (SP) fused to *gfp* (Fig. 3.3 (2b)).

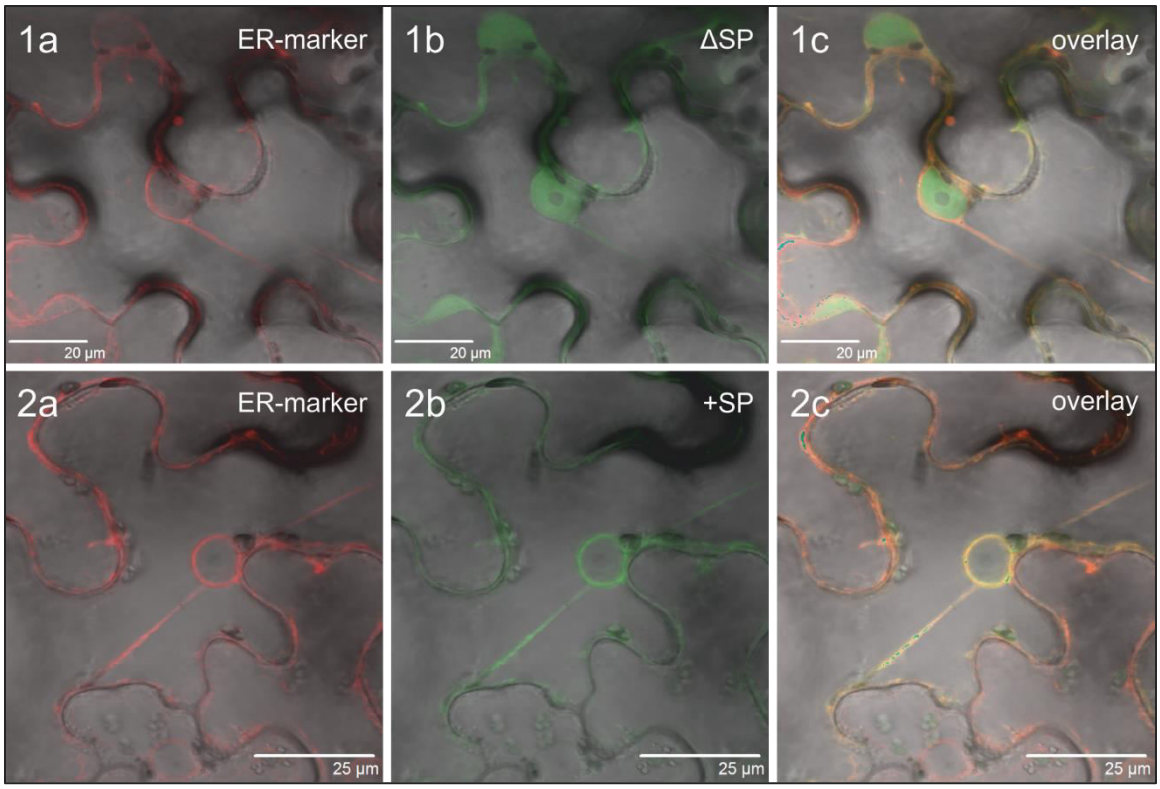

Fig. 3.3: Results of co-localisation studies in *N. benthamiana* (picture taken with a confocal laser scanning microscope, CLSM, Leica TCS SP2). 1: Co-infiltration of ER-marker-mRFP and CeLV ΔSP-GFP, **1a:** ER-marker-mRFP, **1b:** CeLV ΔSP-GFP, **1c:** overlay 1a+1b, **2:** Co-infiltration of ER marker-mRFP and CeLV +SP-GFP, **2a:** ER-marker-mRFP, **2b:** CeLV +SP-GFP, **2c:** overlay 2a+2b

The red fluorescence of the ER-marker-mRFP is visible throughout the cytoplasm and around but not in the nucleus (Fig. 3.3 (1a+2a). In case of the construct without SP (Fig. 3.3 (1b)) the GFP fluorescence appears in the cytoplasm and in the nucleus. The overlay (Fig. 3.3 (1c)) shows clearly that the two reporters do not co-localise completely. Co-localisation is represented by yellow to orange areas. A different picture was seen in the co-localisation experiment of the ER-marker-mRFP (Fig. 3.3 (2a)) in combination with CeLV +SP-GFP (Fig. 3.3 (2b)). The overlay of these pictures show a complete co-localisation of the reporter proteins, resulting in yellow fluorescence in the cytoplasm and the area around but not in the nucleus. These results indicate that the predicted signal peptide is functional and guides proteins to or into the ER.

3.4.2 CeLV and PPV silencing constructs

All silencing constructs of CeLV and PPV used in the experiments are illustrated in figures 3.4 and 3.5.

In case of PPV, the protein identities and cleavage sites in the polyprotein are known so that the constructs are mostly designated with the protein identity. For CeLV, less is known about the putative cleavage sites and protein identities in the N-terminal region. Because of that, the constructs are not designated with proteins name but first and/or last amino acids. For example, the counterpart of CeLV VRNC-AIVG is PPV HC-Pro (HC-Pro without P1). For a better understanding, the CeLV constructs and matching PPV constructs are listed in table 3.1.

Tab. 3.1: CeLV constructs and their PPV counterparts.

Silencing construct CeLV	Amino Acid position (polyprotein)	Silencing construct PPV	Amino Acid position (polyprotein)
CeLV DPPH-DECH	306-1364	PPV FANT-DECH	239-1345
CeLV AIVG	1-738	PPV P1+HC-Pro	1-766
CeLV DPPH-AIVG	306-738	PPV FANT-HC-Pro	239-766
CeLV SIAT-AIVG	358-738	PPV KVSK-HC-Pro	296-766
CeLV VRNC-AIVG	371-738	PPV-HC-Pro	310-766
CeLV IEKY	1-370	PPV-P1	1-308

In the following figure 3.4 all CeLV silencing constructs are illustrated in comparison to the full-length CeLV polyprotein. All inserts were located downstream of CaMV 35S promoter (and in some cases leader sequence) followed by a HDV ribozyme and a CaMV 35S pA (not integrated in the figures). The leader sequence is represented by a grey box directly before the insert sequence.

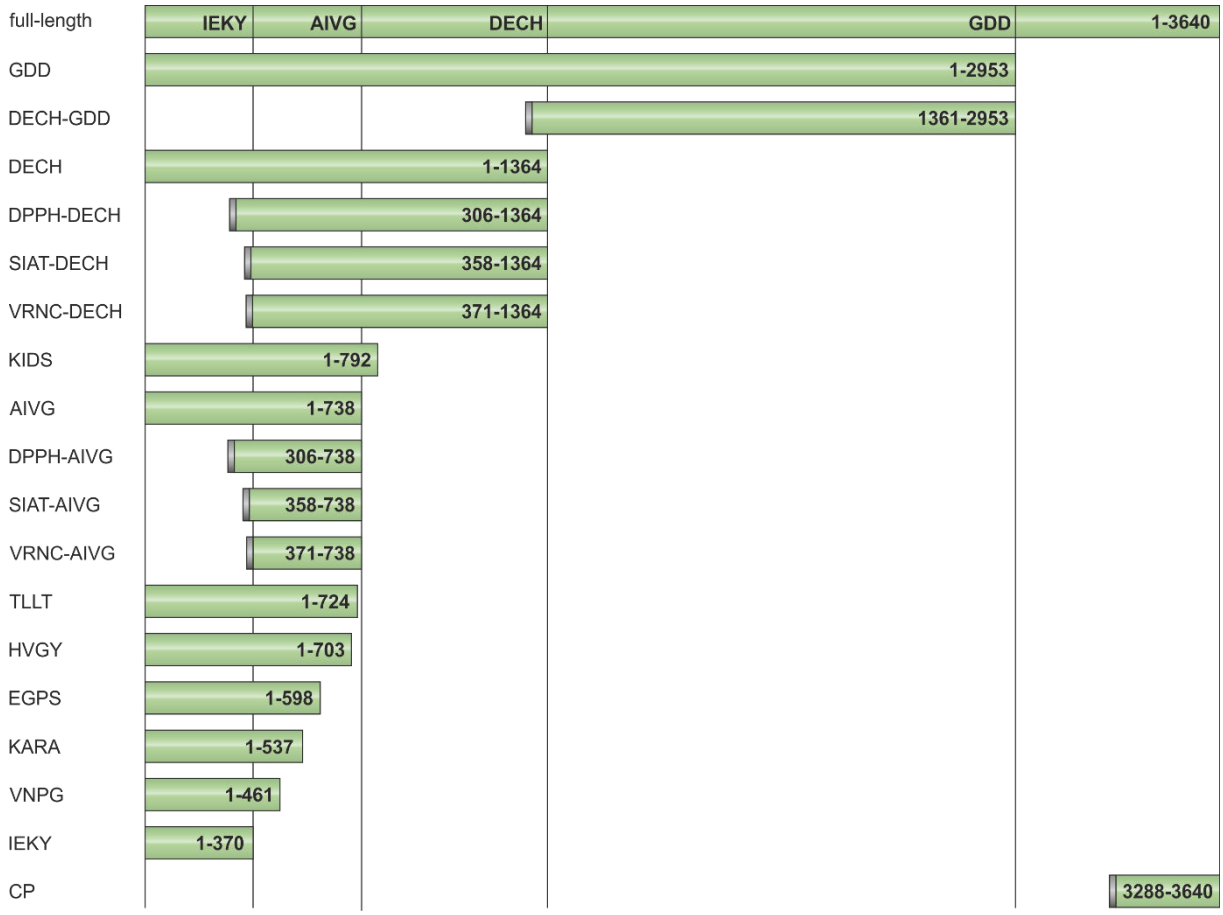

Fig. 3.4: All CeLV silencing constructs compared to the full-length polyprotein sequence. Constructs, which have the leader sequence instead of the viral 5'-UTR own a grey box. Numbers indicate the amino acid positions in the viral polyprotein, which are translated from the insert sequence.

The following figure 3.5 summarizes the constructs designed for PPV.

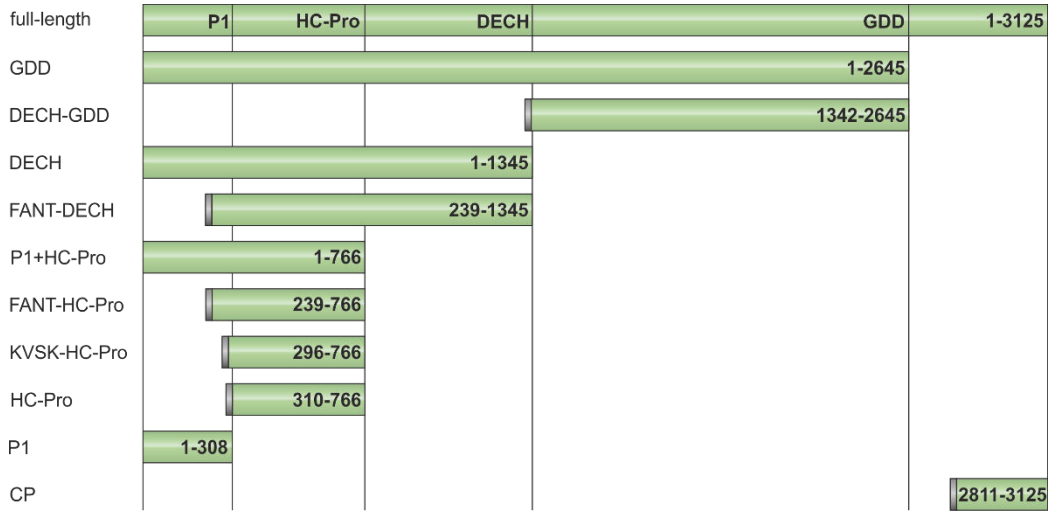

Fig. 3.5: All PPV silencing constructs compared to the full-length polyprotein sequence. Constructs, which have the leader sequence instead of the viral 5'-UTR own a grey box. Numbers indicate the amino acid positions in the polyprotein, which are translated from the insert sequence.

All constructs were sequenced at their ligation sites and the correct insertion could be confirmed.

3.4.3 Local transient silencing assays – establishment and fluorescence development over time

All silencing constructs were transformed into *R. radiobacter* and co-infiltrated with pBI 16cgfp (abbreviated GFP) into leaves of *N. benthamiana* 16c. The GFP fluorescence was documented over certain periods of days to detect putative silencing suppression activities. Within the experiments the reduction and duration of the GFP fluorescence varied strongly, leading to different final time points of documentation (5 dpi – 9 dpi). Selected examples are illustrated in the following figures. All results are summarized in a schematic figure at the end of the chapter (Fig. 3.13+3.14).

Figure 3.6 shows the results for the PPV clones P1, P1+HC-Pro, DECH and GFP as the negative control 3, 6 and 9 days after infiltration.

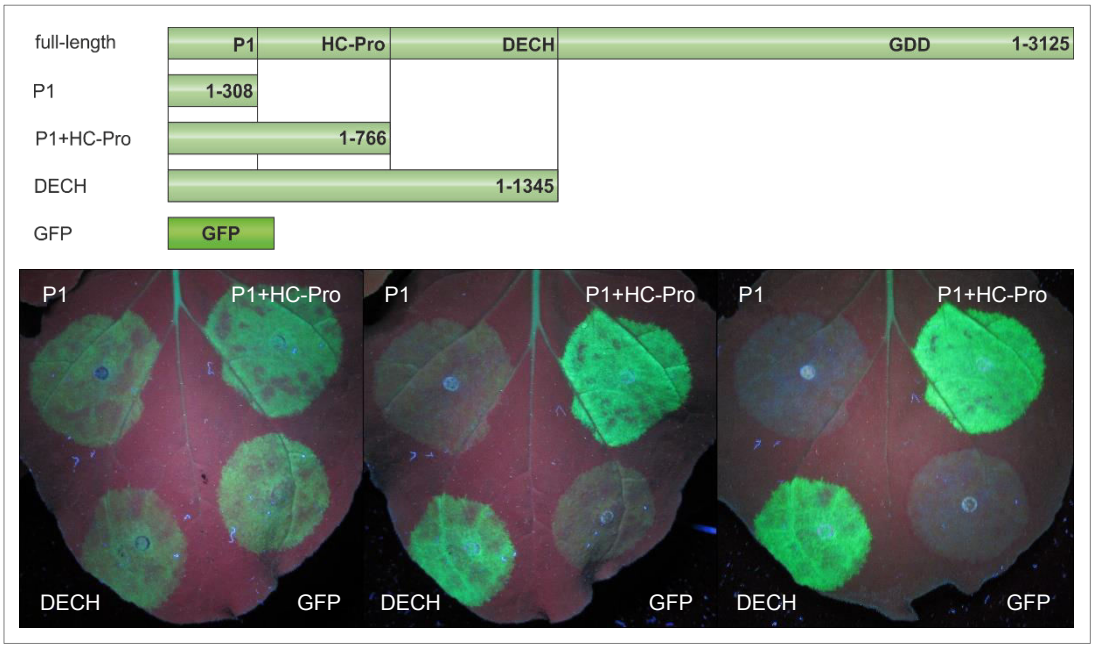

Fig. 3.6: Schematic illustration and local silencing assay of the constructs PPV P1, PPV P1+HC-Pro, PPV DECH and GFP 3, 6 and 9 days after infiltration into a *N. benthamiana* 16c leaf. A PPV full-length clone scheme is added as opportunity to compare the construct lengths. The PPV full-length clone is not part of the local silencing assay.

Three days after infiltration no differences in fluorescence intensity between the clones could be seen but with increasing duration of the experiment differences were observed. The GFP fluorescence in the spots containing PPV P1+HC-Pro and PPV DECH was stable and of same intensity after 9 days. These two clones include

the known silencing suppressor of potyviruses, the HC-Pro. In contrast, a clear reduction in the fluorescence was seen for PPV P1 and GFP over time, representing the expectations since no suppressor activity is known for P1 or GFP. The red halo around the spots P1 and GFP indicate the short-distance mobile silencing.

The results for the corresponding silencing clones of CeLV are presented in the next figure 3.7. GFP fluorescence was documented 3, 6 and 9 days after infiltration.

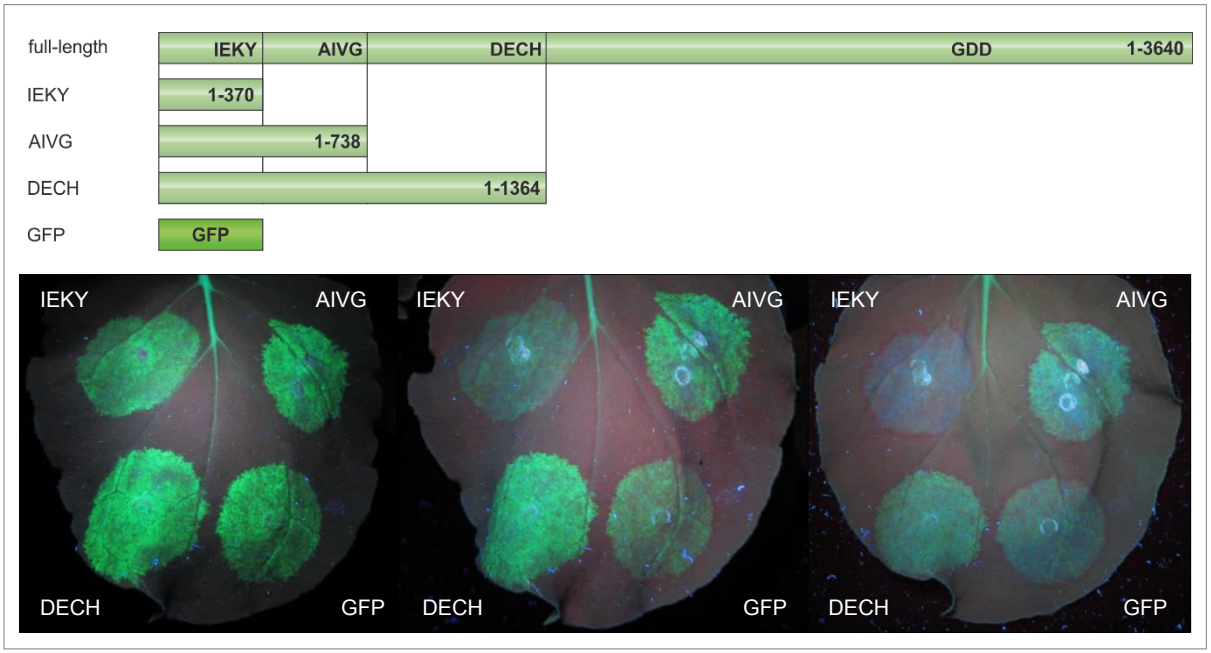

Fig. 3.7: Schematic illustration and local silencing assay of the constructs CeLV IEKY, CeLV AIVG, CeLV DECH and GFP 3, 6 and 9 days after infiltration into a *N. benthamiana* 16c leaf. A CeLV full-length clone scheme is added as opportunity to compare the construct lengths. The CeLV full-length clone is not part of the local silencing assay.

In the beginning of the experiment (3 dpi) there were no differences in fluorescence intensity detectable compared to the PPV experiment (Fig. 3.6) as well as between the different clones. Nine days after infiltration, CeLV IEKY showed the weakest signal, followed by GFP with a stronger fluorescence than in the experiment before. This effect was observed in other experiments, too, so that the GFP control was replaced by CeLV IEKY or CeLV CP. In this experiment every spot showed a red halo. Infiltration of CeLV AIVG and CeLV DECH, both containing the suspected region of a putative suppressor protein, resulted in a higher intensity, indicating a possible suppression activity. Overall, the PPV clones show a higher fluorescence intensity over time than the CeLV clones.

For direct comparison, CeLV (C-) AIVG and C-DECH were infiltrated above PPV (P-) P1+HC-Pro and P-DECH in the same leaf (Fig. 3.8).

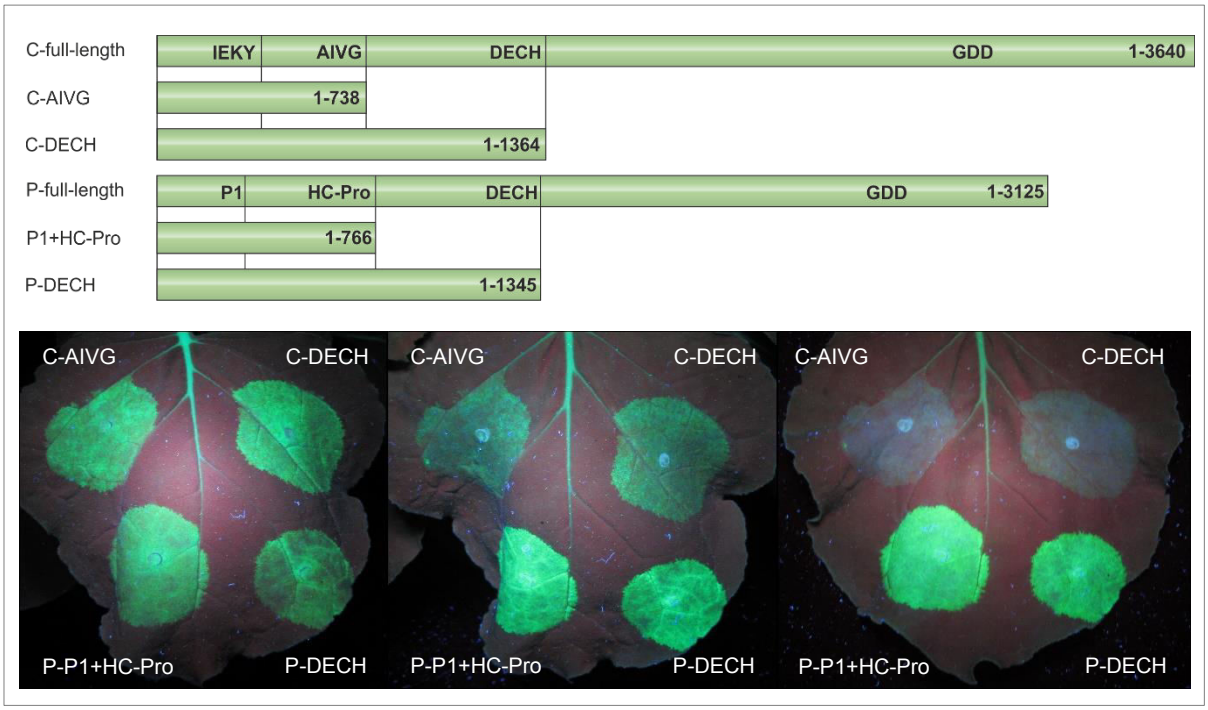

Fig. 3.8: Schematic illustration and local silencing assay of the constructs CeLV (C-) AIVG, C-DECH, PPV (P-) P1+HC-Pro and P-DECH 3, 6 and 9 days after infiltration into a *N. benthamiana* 16c leaf. A CeLV and a PPV full-length clone scheme is added as opportunity to compare the construct lengths. Both full-length clones are not part of the local silencing assay.

In this experiment the duration of the GFP fluorescence of the CeLV clones is similar to PPV at 3 dpi but a decline was observed at 6 dpi. After nine days the fluorescence of C-AIVG and C-DECH is almost completely gone and the spots are surrounded by a narrow red area, whereas P-P1+HC-Pro and P-DECH appear even more intense as at 3 dpi indicating a stronger suppressor activity. Overall, the local silencing assay worked for PPV and CeLV so that additional constructs were designed.

3.4.4 Studies of further 3' shortened CeLV clones

In order to determine how far the polyprotein can be shortened but at the same time retains its suppressor function, further deletions of CeLV DECH and CeLV AIVG were performed. The following figures show the results of CeLV KIDS, CeLV TLLT and CeLV HVGY, which showed clear fluorescence signals over time in every local silencing assay. CeLV KIDS is 54 amino acids longer than CeLV AIVG and ends directly before PIPO, excluding a putative influence of the latter. CeLV TLLT and CeLV HVGY are 14 and 35 amino acids shorter than CeLV AIVG.

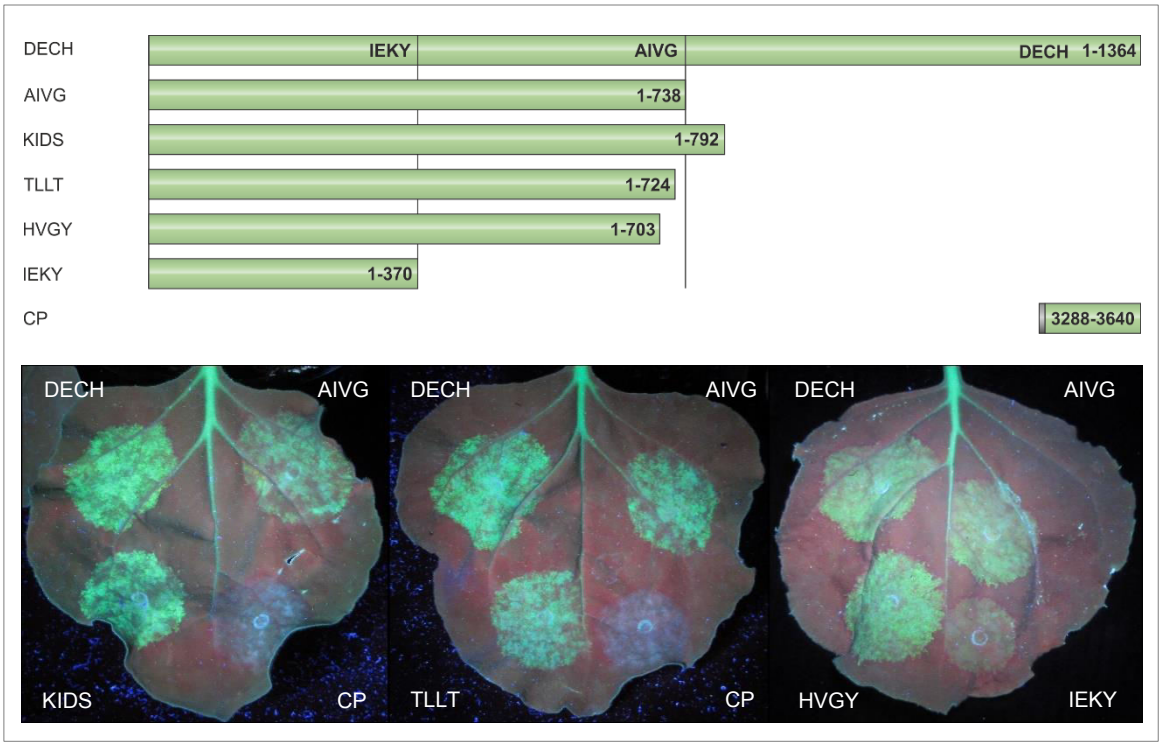

Fig. 3.9: **Schematic illustration and local silencing assay of the constructs CeLV KIDS, CeLV TLLT and CeLV HVGY (7 dpi) compared to CeLV DECH, CeLV AIVG and CeLV CP after infiltration into a *N. benthamiana* 16c leaf.**

In all three results the silencing construct of interest showed an increased fluorescence, compared to the negative controls CeLV CP and CeLV IEKY, however, CeLV IEKY showed a weak fluorescence. The effect, that the negative controls or *R. radiobacter* without a silencing construct (data not shown) sometimes produced a weak GFP signal, could be observed several times. This indicated other parameters influencing the response of the plants to the treatments.

As a consequence of the positive results of CeLV KIDS, CeLV TLLT and CeLV HVGY, additional shorter clones were generated named CeLV EGPS, CeLV KARA and CeLV VNPG. CeLV EGPS is 140 amino acids shorter than CeLV AIVG and the difference to CeLV HVGY is 105 amino acids. CeLV KARA differs from CeLV AIVG and CeLV HVGY in 201 and 166 amino acids and CeLV VNPG in 277 and 242 amino acids respectively. The following selection of results is intended to give an overview about the striking diversity in fluorescence signals between the experiments, exemplified with KARA1-4 and VNPG1-4.

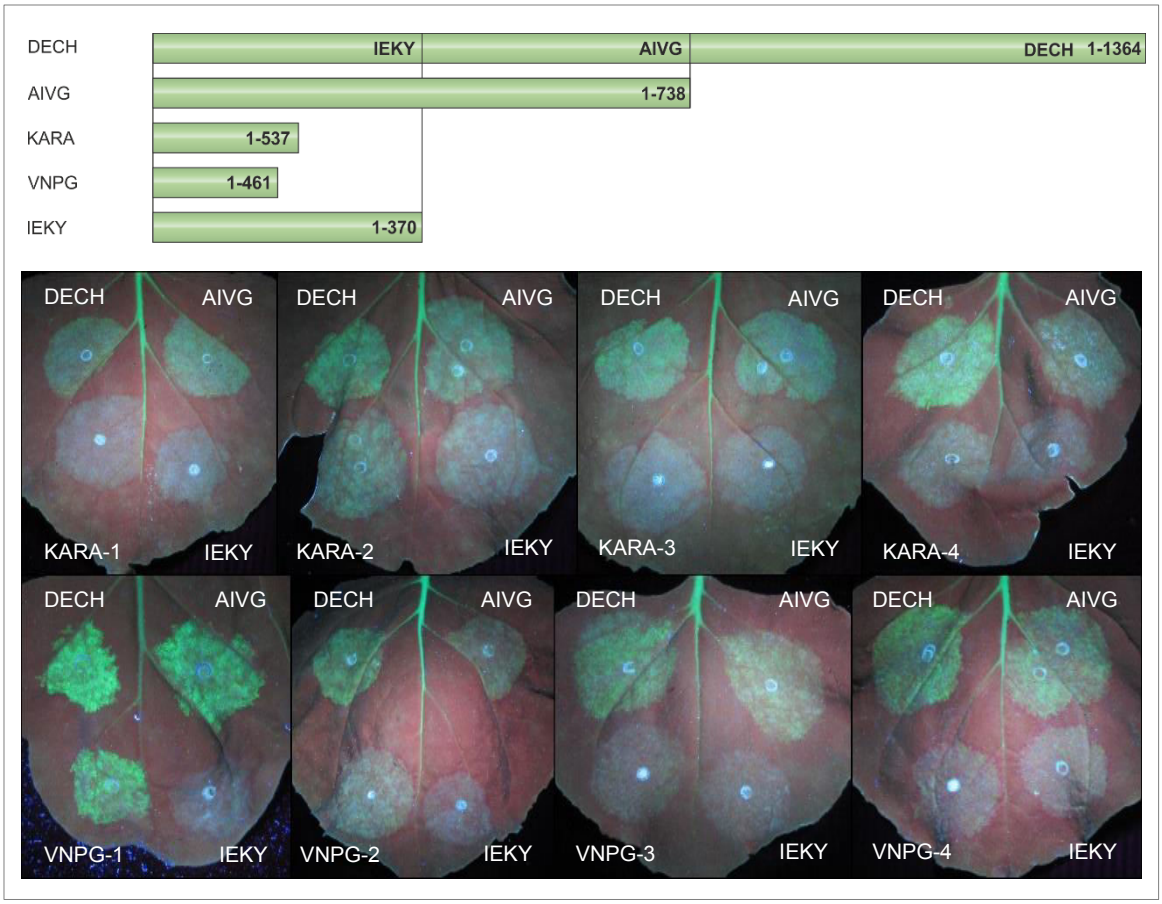

Fig. 3.10: Schematic illustration and local silencing assay of the constructs CeLV KARA-1-3 (7 dpi), KARA-4 (6 dpi) and VNPG-1-4 (7 dpi) compared to CeLV DECH and CeLV AIVG after infiltration into a *N. benthamiana* 16c leaf. The leaves originated from four different repetitions. **1:** VNPG-1; **2:** VNPG-2+3; **3:** KARA-1-3 & VNPG-4; **4:** KARA-4

The spots on these leaves show different fluorescence intensities between different clones but also between same clones within same and different repetitions. For example, the GFP signals of CeLV AIVG and CeLV DECH in VNPG-1 were more intense than in KARA-1. The difficulties in evaluating the constructs of interest as positive or negative can be seen in both cases. KARA-2 and KARA-4 seemed to give a stronger fluorescence than the respective negative control CeLV IEKY but KARA-1 and KARA-3 did not show the same effect. In case of CeLV VNPG, which is 76 amino acids shorter than CeLV KARA, the variances between the repetitions are even stronger. VNPG-1 would be considered as clearly positive as well as VNPG-2, which was already not that intensive. VNPG-3, which originated from the same experiment but other plant than VNPG-2, is not distinguishable from the negative control and same applies to VNPG-4 coming from an additional repetition. The results for CeLV EGPS resembled those of CeLV KARA and CeLV VNPG (data not shown). However, an interpretation of the results needs to take into account the GFP variations of

CeLV KARA and CeLV VNPG compared to each other and CeLV IEKY. No conclusive assessment is possible for these clones. Furthermore, there are no corresponding PPV clones, which could give information about the maximum number of deletable amino acids but still keeping the suppressor activity of the truncated HC-Pro.

3.4.5 Studies of further 5' shortened CeLV & PPV clones

In this subchapter, the suppressor active regions within CeLV and PPV should be defined by deleting amino acids from the N-terminal direction. For this, the clones giving the best fluorescence were chosen, which are PPV P1+HC-Pro, PPV DECH, CeLV AIVG and CeLV DECH.

The first results show the fluorescence signals of three PPV clones, FANT-DECH, FANT-HC-Pro and KVSK-HC-Pro compared to CeLV DECH and the negative controls CeLV IEKY or CeLV CP from two different experiments (Fig. 3.11). The FANT constructs start with the last 70 amino acids of the P1 and KVSK with the last 13 amino acids followed by the rest of the polyprotein until HC-Pro.

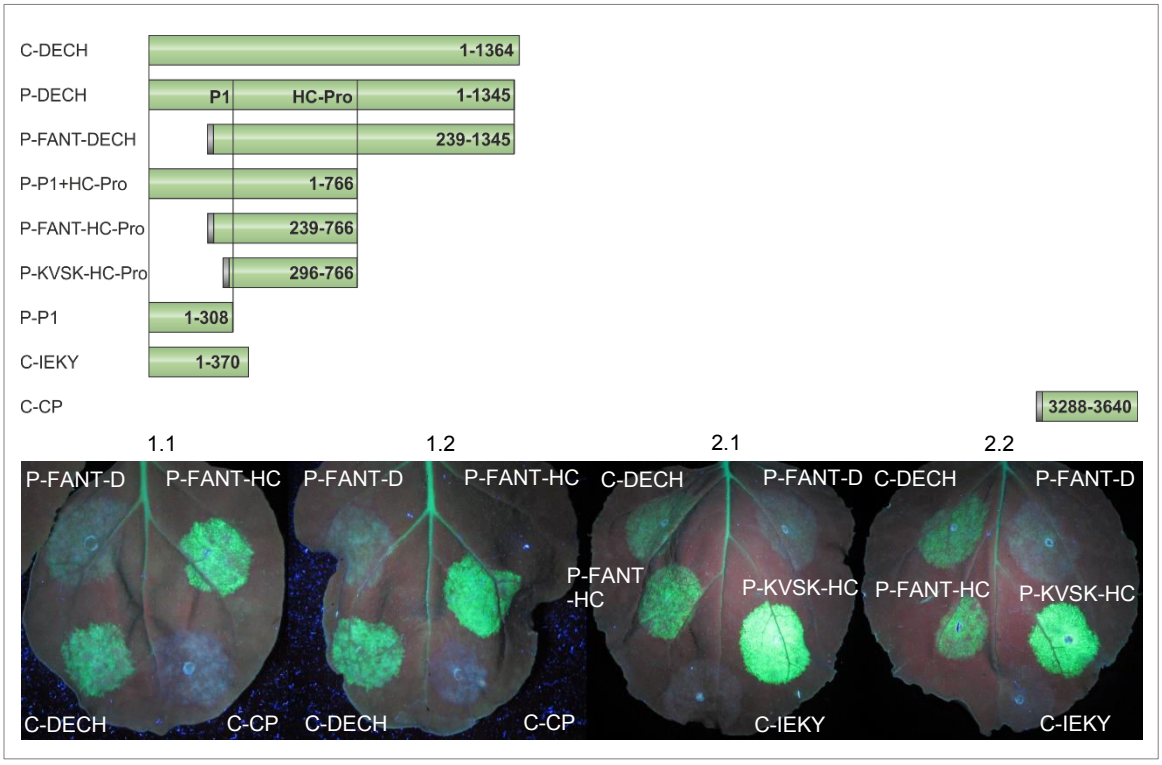

Fig. 3.11: Schematic illustration and local silencing assay of the constructs PPV FANT-DECH (P-FANT-D), PPV-FANT-HC-Pro (P-FANT-HC) and PPV KVSK-HC-Pro (P-KVSK-HC) (7 dpi) compared to CeLV DECH (C-DECH), CeLV CP (C-CP) or CeLV IEKY (C-IEKY) after infiltration into a *N. benthamiana* 16c leaf. The leaves originated from two different repetitions and different plants (1&2).

In this experiment it points out, that PPV FANT-DECH did not show any silencing suppression activity in comparison to CeLV DECH although the HC-Pro region is entirely present. In contrast, PPV FANT-HC-Pro and KVSK-HC-Pro show a clear and intense fluorescence indicating that the N-terminal remaining amino acids did not have a negative influence of the functionality. Complete sequencing of PPV FANT-DECH revealed an adenine insertion at position 2,809 (counted from the ATG) in the CI coding sequence leading to premature stop codons in the following sequence. A nucleotide exchange C236G in the HC-Pro led to a S79L amino acid exchange.

This strategy was also applied for CeLV and the results are illustrated in figure 3.12. CeLV VRNC-DECH (VRNC-D) represents the putative HC-Pro like protein solely and DPPH-DECH (DPPH-D) and SIAT-DECH (SIAT-D) have N-terminal extensions of 65 and 13 amino acids. The results originated from different experiments and plants.

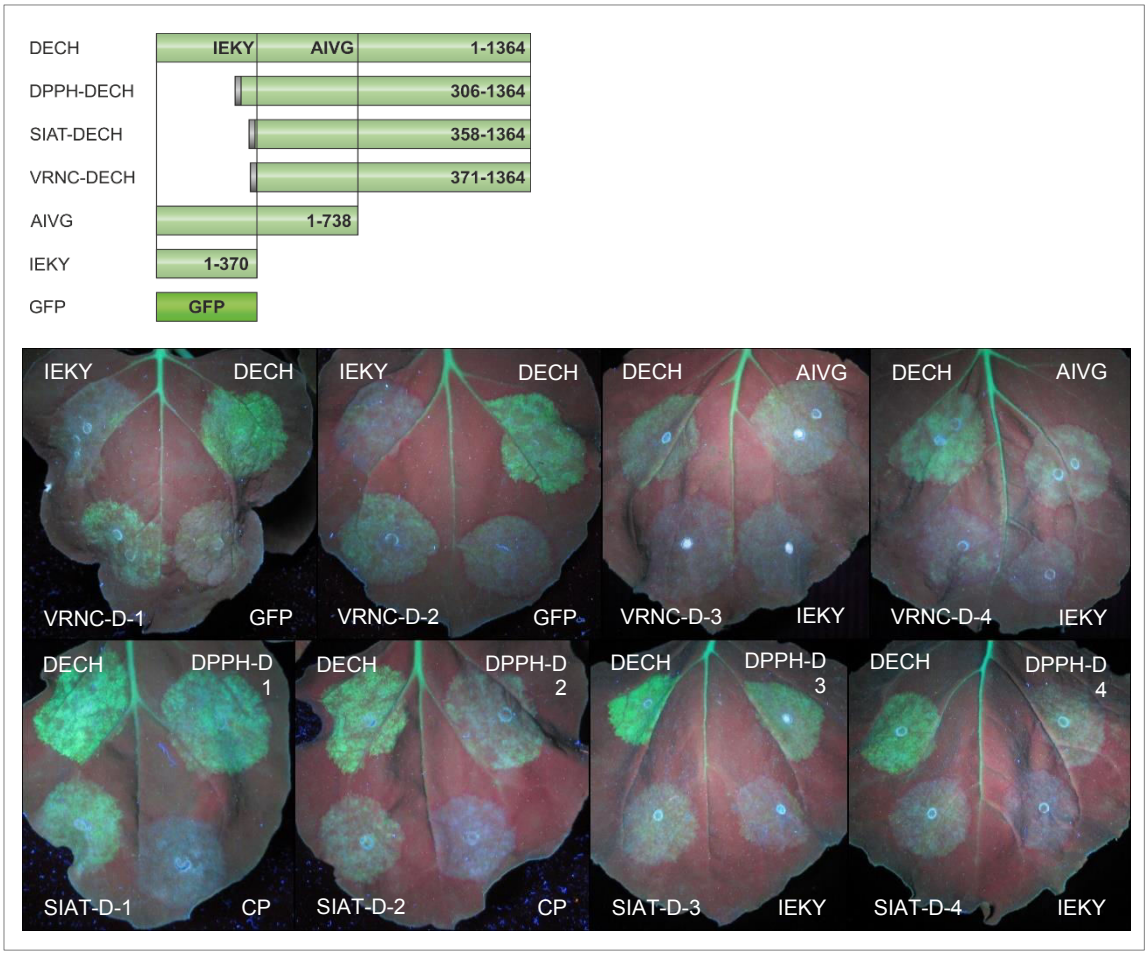

Fig. 3.12: Schematic illustration and local silencing assay of the constructs CeLV VRNC-DECH (VRNC-D)-1 (6 dpi), VRNC-D-2 (9 dpi), VRNC 3+4 (7 dpi), CeLV DPPH-DECH (DPPH-D) 1-4 and CeLV SIAT-DECH (SIAT-D) 1-4 (7 dpi) compared to CeLV DECH and CeLV AIVG after infiltration into a *N. benthamiana* 16c leaf. The leaves originated from five different repetitions. **1:** VRNC-D-1; **2:** VRNC-D-2; **3:** DPPH&SIAT-D-1+2, **4:** DPPH&SIAT-D-3+4; **5:** VRNC-D-3+4

As already seen in 3.4.4 the fluorescence intensities vary dramatically. In case of VRNC-D1 and 2 the spot could be rated positive whereas VRNC-D3 and 4 were not distinguishable from the negative controls. The results of VRNC-AIVG resembled those of the DECH variant (data not shown). For DPPH-D and SIAT-D the same problem occurred. The first result (1) seemed to be positive but already the second leaf (2) showed a less intensive GFP signal even though they originated from the same experiment. During an additional experiment the fluorescence decreased and it is questionable whether these constructs could be evaluated as positive or negative. The results for the CeLV AIVG variants of DPPH and SIAT are not illustrated because the difficulties were the same.

Overall, the fluorescence intensities of the different clones varied extremely and in some cases even CeLV AIVG did not reveal GFP fluorescence anymore. As a consequence, the evaluation of some clones was challenging and sometimes not possible. Compared to the HC-Pro of PPV, the putative silencing suppressor of CeLV seems to be less strong and was not able to reach such high GFP fluorescence intensities over time. To summarize the experiments, a schematic overview of all clones and their ability to suppress the gene silencing is given in figures 3.13 and 3.14.

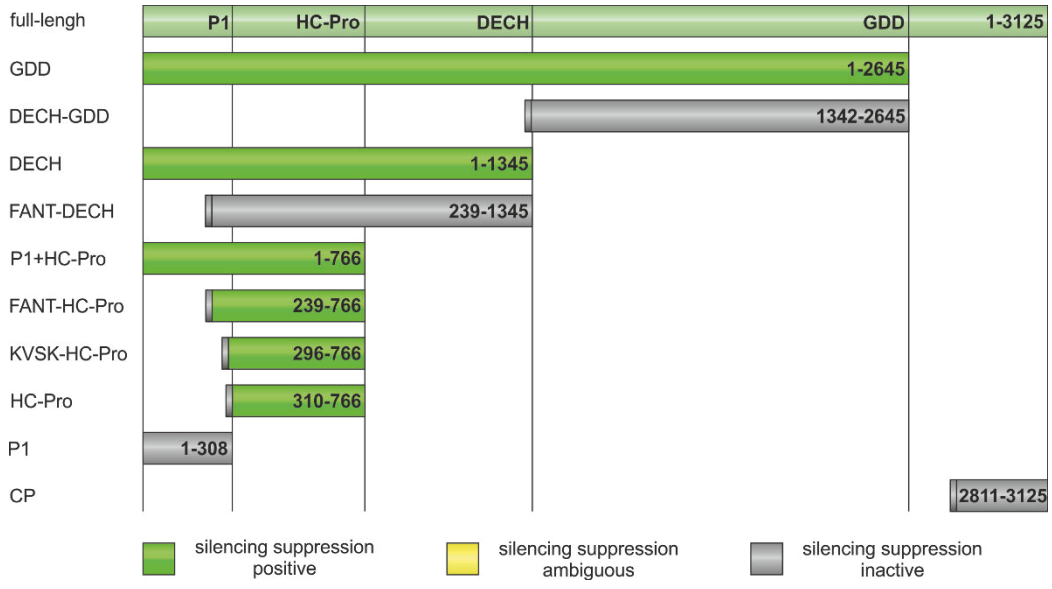

Fig. 3.13: Schematic overview of the PPV silencing constructs compared to the full-length polyprotein sequence and their ability to suppress the gene silencing *in planta*. Constructs, which have the leader sequence instead of the viral 5'-UTR own a grey box. Numbers indicate the amino acid positions in the viral polyprotein which are translated from the inserted sequence. The colour of the bars indicate the suppressor activity: **green:** positive, **yellow:** ambiguous and **grey:** no activity

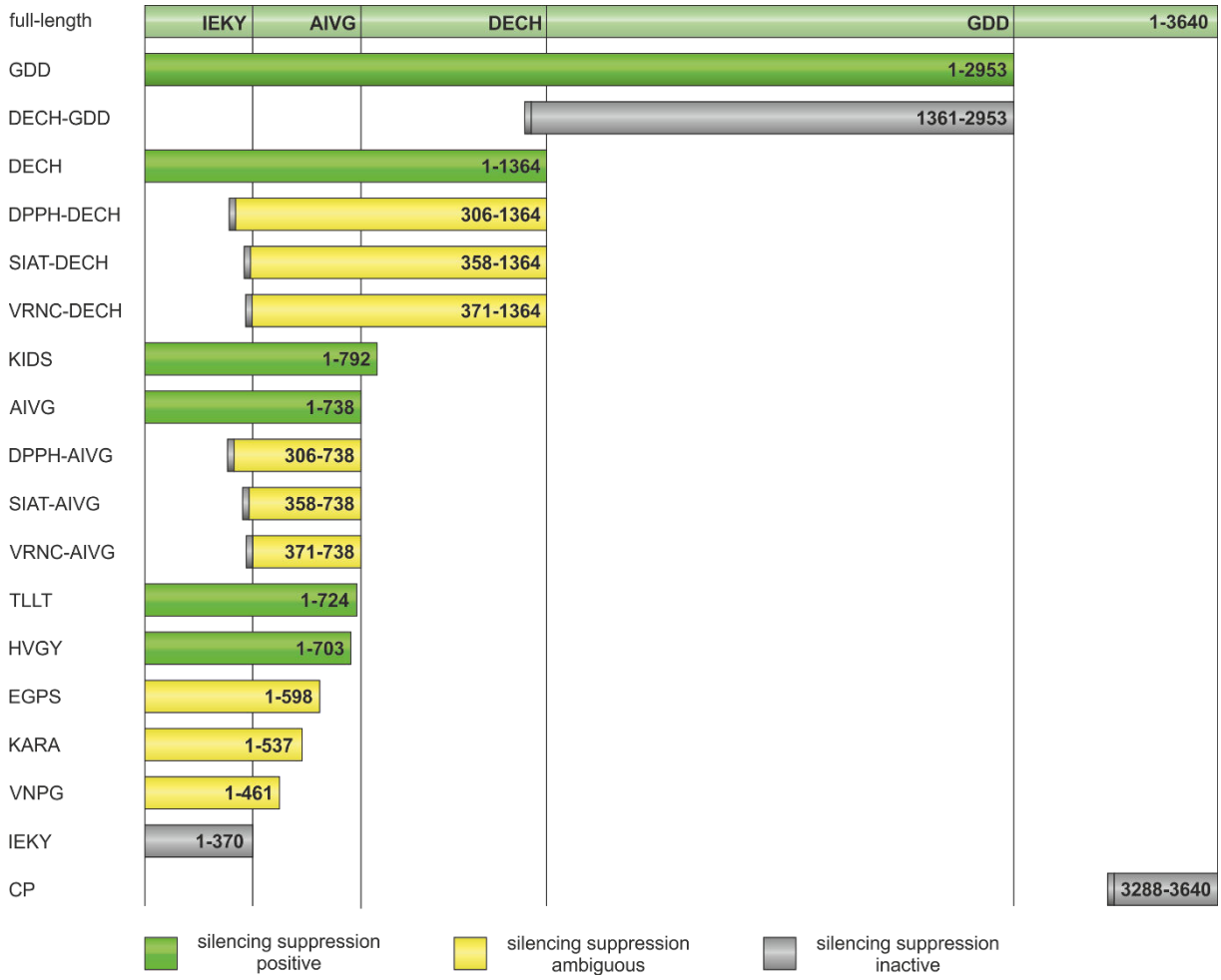

Fig. 3.14: Schematic overview of the CeLV silencing constructs compared to the full-length polyprotein sequence and their ability to suppress the gene silencing *in planta*. Constructs, which have the leader sequence instead of the viral 5'-UTR own a grey box. Numbers indicate the amino acid positions in the viral polyprotein which are translated from the inserted sequence. The colour of the bars indicate the suppressor activity: **green:** positive**, yellow:** ambiguous and **grey:** no activity

From these findings it can be concluded that CeLV has a silencing suppressor which is located within the first 703 amino acids of the polyprotein (CeLV HVGY) being less strong compared to the HC-Pro of PPV.

3.5 Discussion

3.5.1 The putative signal peptide guides proteins to the ER

In the co-localisation studies it could be clearly demonstrated, that the predicted signal peptide is functional and leads proteins to the endoplasmic reticulum (Fig 3.3). To our knowledge, this genomic element is unique in the family *Potyviridae*. Signal peptides share a common structure of a cytoplasmic positively charged N-region of one to five

amino acids in length, connected to a hydrophobic region consisting of seven to 15 amino acids. An additional region being three to seven amino acids in size (C-region) is adjacent at the C-terminal position and contains the cleavage site of the signal peptidase (Heijne 1982; Auclair et al. 2012). The amino acids around the cleavage site follow the so-called “-1,-3 rule”. It says that positions -1 and -3 are mostly composed of small and neutral amino acids (e. g. A, C, G, S and T) (Heijne 1983). In case of the CeLV signal peptide these characteristics can be applied so that the cleavage by a signal peptidase is highly probable but was not investigated in this study.

Rotaviruses have a dsRNA genome and consist of eleven segments. Their outer capsid glycoprotein VP7 was shown to have an N-terminal located signal peptide which leads the protein to the ER where it retains in a membrane-associated manner (Stirzaker, Both 1989). Flavivirids ((+)ssRNA genome) were shown to have different kinds of C-terminal located signal peptides which are used for releasing their membrane-associated glycoproteins from the polyprotein. In case of *Hepatitic C virus*, four cleavages mediated by the ER signal peptidase and one by the ER signal peptide peptidase are known (Moradpour, Penin 2013).

For plant viruses, putative signal peptides of 24 to 35 aa have been shown in the glycoproteins (M RNA segment) of the tospoviruses bean necrotic mosaic virus (BeNMV) and TSWV for example (Kormelink et al. 1992; Oliveira et al. 2012). They play a role as a leader for glycoproteins to and from the ER to the Golgi (Ribeiro et al. 2008; Chen et al. 2012). Other studies on *Tomato ringspot nepovirus* (ToRSV) revealed a signal peptide and cleavage by a signal peptidase in the NTB-protein C-terminal half. Is was found out, that the VPg domain, containing a putative glycosylation site N-X-T, was translocated into the ER lumen and glycosylated using *in vitro* membrane-associated modification assays combined with deglycosylation assays. (Han, Sanfaçon 2003; Wang et al. 2004; Wei et al. 2016). Analysis of the first 370 amino acids of the CeLV polyprotein (a putative P1-like protein) with NetNGlyc 1.0 Server (Gupta et al. 2004) revealed two possible N-glycosylation sites, following the N-X-S/T pattern, whereof the first is highly possible (N-L-T, positions 51-53) and the second (N-N-S, positions 226-228) less possible to be glycosylated. If it is assumed, that the signal peptide guides the first protein into the ER and perhaps to the glycosylation pathway, detailed information about its potential functions are needed. Furthermore, it has to be investigated if more than the first protein is translocated. It is

known, that P1 of potyviruses interacts with proteins of the replication complex for example P3 (*in vitro* only) and CI *in vitro* and *in planta* indicating a possible role in virus replication but nothing about ER retention and/or glycosylation is known (Merits et al. 1999; Zilian, Maiss 2011). Other studies reported P1 located in the nucleolus and interacting with 60S ribosomal subunit (Martinez, Daros 2014). Presumably, CeLV contains an uncommon variant of a protein in the first position of the polyprotein. Concerning potyviral ER targeting, it is known that the 6K2 protein is located at ER membranes and ERES (endoplasmic reticulum exit sites) and induces formation of ER-derived vesicles for the replication complex (Schaad et al. 1997a). From the ER, the vesicles move to the outer chloroplast envelope (Wei et al. 2010a). It could be possible that the signal peptide of CeLV resembles this function. In this context, the elucidation of the functions of all the N-terminal located proteins is of great importance.

3.5.2 A suppressor of post-transcriptional gene silencing is located in the N-terminal region of the CeLV polyprotein

Sequence analyses of CeLV did not reveal any of the known conserved motifs of a HC-Pro or another potential silencing suppressor but due to the similarities to the *Potyviridae* family, it was assumed that a suppressor protein is located in the first third of the genome. Investigations on the presence of a putative silencing suppressor were performed using a transient local silencing assay in which partial viral genome segments are cloned and co-infiltrated with GFP into a leaf of a GFP transgenic *N. benthamiana* 16c line. This method was used in many studies to identify potential suppression activities of viral proteins like CVYV (P1b), peanut clump pecluvirus (PCV, p15), grapevine vitivirus A (GVA, p10) and tomato yellow leaf curl begomovirus (TYLCD, C2, C4, V2) (Dunoyer et al. 2002; Valli et al. 2006; Zhou et al. 2006; Luna et al. 2012). With this method it was possible to confirm the presence of a silencing suppressor within the CeLV genome and to narrow down the section of the active region to the amino acids 1 to 703 of the polyprotein. The mobile silencing signal could not be interrupted by this protein indicated by the deep red area around the infiltration spots. This result is consistent with the current knowledge about potyviral silencing suppressors being unable to stop the mobile silencing signal indicating a similar mode of action (Mallory et al. 2001). The fact that CeLV IEKY did not show any fluorescence does not allow to assign the silencing active region between the amino acids 371 and 703. It could be possible, that the predicted cleavage site IEKY/V is incorrect. Thus,

the constructs CeLV IEKY and VRNC AIVG/DECH represent incomplete proteins either at the C-terminus or the N-terminus, which are not functional anymore.

Unfortunately, it was not possible to draw conclusions concerning the number and exact cleavage sites of proteins by studying the silencing suppression activity. Including all gathered information, the following figure summarizes the most possible N-terminal compositions, which are conceivable for the CeLV polyprotein (Fig. 3.15).

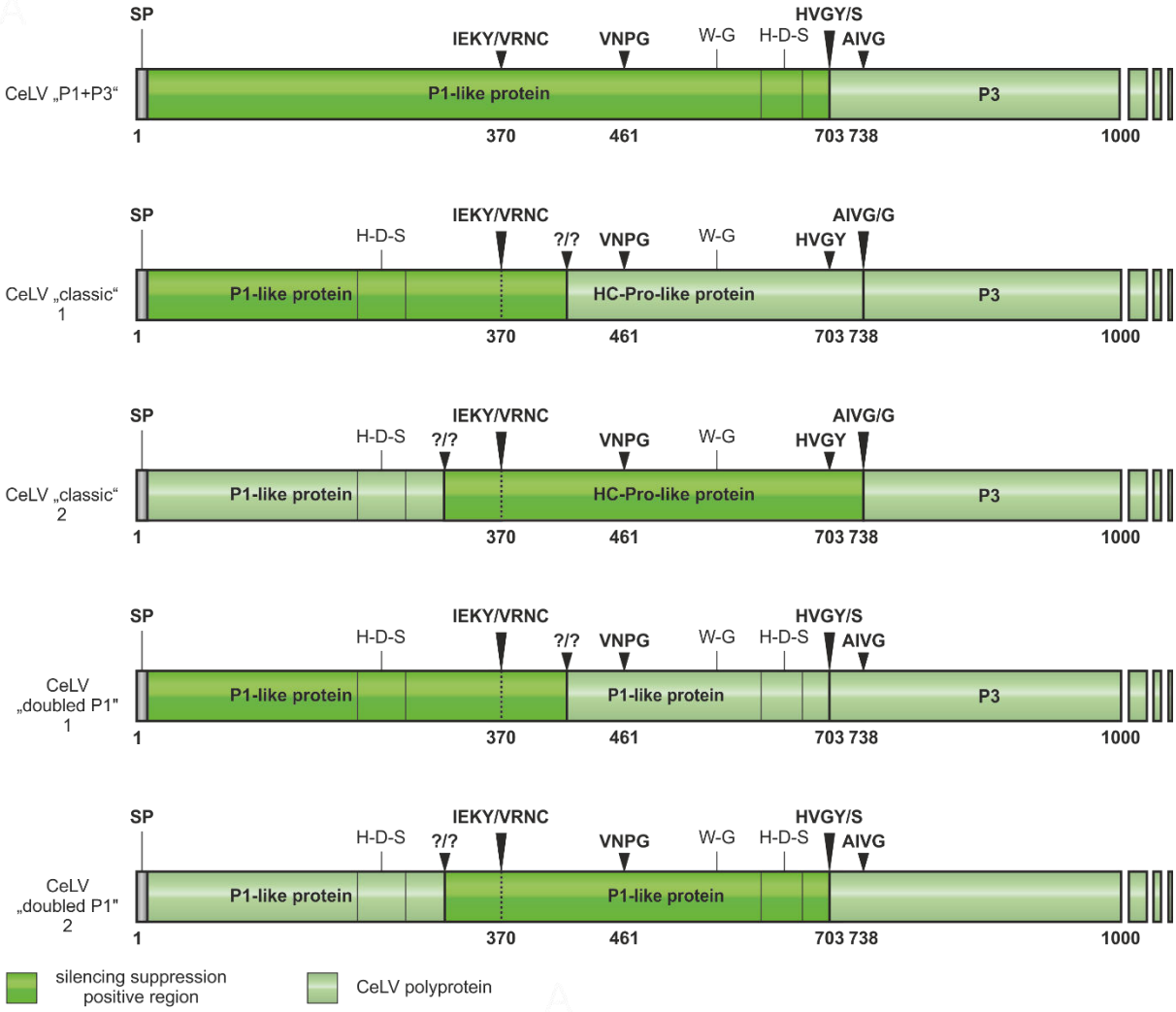

Fig. 3.15: Schematic model of the conceivable N-terminal compositions of CeLV. The numbers under the bars are the amino acid positions within the polyprotein**. Intense green** regions mark silencing activity. **Long black filled triangles** indicate putative protease cleavage sites suitable for the respective model. **Small black filled triangles** are other motifs used for some constructs as orientation or unknown **(?/?)**. **Clear boxes and "H-D-S"** mark the possible catalytic triad which is typical for P1-like serine proteases. **"WG"** marks a putative "AGO-Hook"-motif.

The models CeLV "P1+P3", CeLV "classic 1&2" and CeLV "doubled P1 1&2" seem to be the most likely ones, referring to two possible catalytic triads and/or putative protease cleavage sites within the polyprotein. CeLV "P1+P3" takes into account the

possibility of a genomic composition like CBSV and UCBSV with a missing HC-Pro-like protein and a large P1 followed directly by P3. CeLV “classic 1 & 2” refer to a potyvirus-like genome organization with a P1-like protein and a HC-Pro-like protein. Another possibility is the presence of two P1-like proteins, illustrated in CeLV “doubled P1 1&2”, resembling the genomes of CVYV and SqVYV.

CeLV “P1+P3” would fit best with the results obtained in the experiments because a clear fluorescence intensity was observed for the construct CeLV HVGY which comprises the first 703 amino acids of the polyprotein (Fig. 3.9, right). CeLV “classic 1” and CeLV “doubled P1 1” propose the first of two proteins to be the silencing suppressor. This option would be possible, if the clones EGPS, KARA, and VNPG turn out to have a silencing active region, which could not be verified in this study due to ambiguous results in the experiments (Fig. 3.10). To date it is not clear if the CeLV silencing suppressor, which seems to be less active than the PPV HC-Pro (Fig. 3.8), shows enough activity to be recognized by this method, especially when the correct cleavage site is not targeted in a construct (Fig. 3.15, indicated with “?/?”). This would result in C-terminal remaining amino acids, which might lead to a decreased activity. It has to be investigated if a truncated HC-Pro of PPV with a certain deletion of the C-terminus is still able to suppress the gene silencing. If the putative analogous CeLV protein would react the same way is not clear.

The models CeLV “classic 2” and CeLV “doubled P1 2” show a potential silencing suppressor located in the second position of the polyprotein of CeLV. In this case, it is of great importance to determine if the DPPH- and SIAT-constructs are definitely negative or positive. It could be shown, that PPV FANT-DECH is not functional but PPV FANT-HC-Pro and KVSK-HC-Pro (Fig. 3.11). It could be speculated, that the 70 N-terminal remaining amino acids in FANT-DECH had a negative effect on the correct folding and cleavage activity of the HC-Pro and that the exact cleavage site has to be targeted. The detected adenine insertion is unlikely to be the reason because it is located in the CI, not affecting the HC-Pro region. Additional constructs like KVSK DECH would be needed for a further investigation. Furthermore, it could be the case, that the N-terminal remaining amino acids are still part of the second protein indicating a larger size. The determination of the N-terminal border is still pending. It is questionable, if this method is successful, when the correct cleavage sites in the CeLV polyprotein are not targeted.

P1 had attracted attention since it had been shown to own W-G/G-W motifs which are able to interrupt the silencing suppression by interfering with AGO1, thus, interacting with RISC. The ipomovirus SPMMV P1 functions in this way and evidence was given that three N-terminal W-G/G-W-motifs are important (Giner et al. 2010). In case of sweet potato feathery mottle virus (*Potyvirus*), an additional overlapping ORF within the P1 coding sequence was detected, spanning most of the P1. This ORF was named PISPO (petty interesting sweet potato *Potyvirus* ORF) and it also contains three W-G/G-W-motifs. Here it was found out, that P1 and PISPO both functioned as a suppressor of silencing and that only the W-G/G-W-motif in the P1 N-terminus but not the three motifs in the PISPO ORF were essential for silencing suppression (Untiveros et al. 2016). In the relevant CeLV sequence, one W-G motif is found located at positions 589 to 590 (C-terminal of the putative second protein) in the polyprotein and hence present in the 3'-shortened silencing constructs CeLV GDD, CeLV DECH, CeLV KIDS, CeLV AIVG, CeLV TLLT, CeLV HVGY and CeLV EGPS. All these clones but CeLV EGPS showed a clear silencing suppression activity and the results of constructs which lack W-G were not reliable (CeLV KARA, CeLV VNPG) or negative (CeLV IEKY). There has as yet been no confirmation of a possible influence of this motif. This could be done by for example replacement of alanine instead G and/or W resembling the strategy of Untiveros and colleagues (Untiveros et al. 2016). Furthermore it has to be investigated, if the signal peptide could have an impact on this experimental setup by deleting the corresponding region or replacing functional important residues by alanine. In combination with the presence of the signal peptide there are perhaps more possibilities with yet unknown elements and functions concerning silencing suppression mechanisms, which still have to be elucidated.

Due to limitations of the method referring to extreme variations in fluorescence intensities which could possibly be traced back to changing environmental conditions in the greenhouse (drought stress and temperatures over 40 °C in spring and summer months), it was not able to do a more precise determination of a silencing active region. To avoid these problems in future, the experiments have to be adapted to a climate chamber setting to guarantee stable environmental conditions and figure out the best conditions for the plants. If the results are still inconclusive, analyses of mRNA levels or siRNA binding ability could serve as an alternative strategy. Since the silencing suppressor would prevent the mRNA to be degraded, a higher level should be detectable compared to a silencing inactive control. This was successfully shown in

experiments with the potyviral HC-Pro of PPV and P1b of CVYV (Varrelmann et al. 2007; Valli et al. 2011).

For further investigations of the silencing suppressor, it is of great importance to know how many proteins of which size are located in the N-terminus and where the exact cleavage sites are located. For example, it is possible to generate C-terminal shortened versions of the CeLV polyprotein for the use in coupled *in vitro* transcription and translation systems to investigate proteolytic activity for estimation of the size and number of proteins located at the N-terminal region of the polyprotein. The suitability of determining cleavage sites by different approaches was successfully applied for potyviruses like tobacco etch virus, tobacco vein mottling virus and potato virus Y (Mavankal, Rhoads 1991; Verchot et al. 1992; Yang et al. 1998). To determine the exact protein cleavage sites, protein sequencing will be necessary. If the number and size of proteins is clear, further experiments can be carried out to determine a silencing suppressor and amino acids motifs involved in this function.

4 Complete genome sequence and construction of an infectious full-length cDNA clone of a German isolate of *Celery mosaic virus*

4.1 Abstract

The complete genome sequence of a German isolate of celery mosaic virus (CeMV, *Potyvirus*) from Quedlinburg (DSMZ PV-1003) was determined (MF962880). It is the second fully sequenced genome of a virus of this species along with a Californian isolate (NC_015393.1). The positive-sense single-stranded RNA is 10,000 nucleotides in length, shows the typical organization of potyviruses but has a shorter PIPO than the CeMV California. Sequence and phylogenetic analyses revealed differences between the isolates of distinct geographical origins possibly arisen from changing environments. In this study an infectious full-length clone was obtained and the infectivity confirmed by *Rhizobium radiobacter* infiltration of *Apium* species.

4.2 Introduction

Celery mosaic virus (CeMV), a member of the genus *Potyvirus* in the family *Potyviridae,* was first described in California, USA by Severin and Freitag in 1935 (Severin, Freitag 1935) in (Xu et al. 2011b) where it caused large economic losses (Severin, Freitag 1938). To date the virus occurs worldwide with reports from England (Pemberton, Frost 1974), Australia (Latham, Jones 2003), Netherlands (Bos et al. 1989), Germany (Chen, Adams 2001), Venezuela (Fernández et al. 2006), Poland (Paduch-Cichal, Sala-Rejczak 2010), Iran (Khoshkhatti et al. 2011) and Egypt (Amal et al. 2012). Depending on the celery species infected plants show symptoms like stunting, leaf deformation, mosaic and vein-clearing and are not marketable. CeMV is able to infect plants of the families *Apiaceae* and, depending on the isolate, those of the *Amaranthaceae* and/or *Solanaceae* (Severin, Freitag 1938; Brandes, Luisoni 1966; Moran et al. 2002; Khoshkhatti et al. 2011; Amal et al. 2012). Apart from a number of partial genome sequences of the 3'-end from different isolates for example from Australia and the Netherlands, the only complete genome sequence available so far

for CeMV is that of a Californian isolate (abbreviated CeMV-Cal, NC_015393.1) (Xu et al. 2011b).

In this study we report the complete sequence of a CeMV isolate from Quedlinburg (MF962880), Germany (DSMZ PV-1003) and the construction of full-length cDNA clone of CeMV that was subsequently shown to be infectious following infiltration of two *A*. *graveolens* cultivars with *R*. *radiobacter*. In a host range test the isolate was not able to infect *Chenopodium* and *Nicotiana* species.

4.3 Material and Methods

4.3.1 Origin of virus isolate, mechanical transmission and host range

CeMV-Quedlinburg (CeMV-Que) was originally collected by Dr. H. J. Vetten and dried plant material of an infected *Ammi majus* plant was kindly provided as virus inoculum by the DSMZ Braunschweig, Germany. The virus was propagated on *A*. *graveolens* var. *rapaceum* “Mars” by mechanical transmission. Therefore, infected plant material was ground in phosphate buffer (0.05 M KH_2PO_4, 0.05 M Na_2HPO_4, 1 mM EDTA, 5 mM Na-DIECA) together with celite and charcoal and rubbed onto two to three mid-aged leaves of a healthy seedling. In order to study the host range of CeMV, three plants of each species were inoculated mechanically with sap from infected *A*. *graveolens*. Additionally, one *A*. *graveolens* plant was inoculated with the virus as a positive control. For detailed information concerning provider of the plant species see table 8.1.

4.3.2 Oligonucleotides used for verification of infection and construction of the infectious full-length cDNA clone

The oligonucleotides for the amplification of the fragments to build the full-length clone were designed on the basis of the complete sequence of the Californian CeMV isolate (NC_015393.1), the ones for the CeMV detection based on sequences of the generated fragments.

Tab. 4.1: Oligonucleotides used for the construction of the infectious full-length cDNA clone and verification of CeMV infection. Underlined parts are vector sequences.

Use of oligonucleotide	Oligonucleotide name	Sequence of oligonucleotide
Amplification of pDIVA for Gibson Assembly	pDIVA_as	CCTCTCCAAATGAAATGAACTTCCTTATA TAG
	pDIVA_s	GGGTCGGCATGGCATCTCCACCTCCTC
Amplification of fragment 1 for Gibson Assembly into pDIVA	CeMV_VKF1s	GTTCATTTCATTTGGAGAGGAAAAACAA AACAAATCATAC
	CeMV_VKF1as	CAACCTAAAGCGCACCATGAATCGTC
Amplification of fragment 2 for Gibson Assembly into pDIVA	CeMV_VKF2s	GACGATTCATGGTGCGCTTTAGGTTG
	CeMV_ VKF2pDIVAas	GAGATGCCATGCCGACCCAGCATGACG CGTGCTTGCTTGACTGTGC
Amplification of plasmid containing fragments 1+2	CeMV_open12as	AGCATGACGCGTGCTTGCTTGACTGTGC
	pDIVA_s	GGGTCGGCATGGCATCTCCACCTCCTC
Amplification of fragment 3 for Gibson Assembly into pDIVA + fragments 1+2	CeMV_VKF3s	AATGCACAGTCAAGCAAGCACGCGTCAT G
	CeMV_VKF3as	GATTCGCTCATTTGATTTCGGTGCTC
Amplification of fragment 4 for Gibson Assembly into pDIVA + fragments 1+2	CeMV_VKF4s	GAGCACCGAAATCAAATGAGCGAATC
	CeMV_VKF4as	GAGATGCCATGCCGACCCGTCTCTTACT ATAAATG
Amplification of defective full-length clone for replacing fragment 1	pDIVA_as	CCTCTCCAAATGAAATGAACTTCCTTATA TAG
	CeMV_VKF2s	GACGATTCATGGTGCGCTTTAGGTTG
Verification of CeMV infection	CeMVdet2s	TGGTTCATGTCACATAAGGGAGTTC
	CeMVdet2as	TTGCGTGCTCTAATAATGGCTTG

4.3.3 Verification of CeMV infection

To confirm an infection, total nucleic acids were extracted using a modified method of Menzel *et al*. (Menzel et al. 2002). For reverse transcription 3 µl of nucleic acid extraction were mixed with 1 µl primer TTGCGTGCTCTAATAATGGCTTG (10 µM; salt free; Eurofins Genomics) and heated at 99 °C for 3 min followed by a rapid cooling on ice. Then, 4 µl 5X RT Buffer (Thermo Fisher Scientific), 0.5 µl dNTPs (10 mM each; Thermo Fisher Scientific), 1 µl RevertAid Reverse Transcriptase (20 U/µl, Thermo Fisher Scientific) and 10.5 µl H_2O were added and incubated at 42 °C for 45 min. For PCR reaction 1 µl cDNA was mixed with 5 µl of FastGene® Taq ReadyMix (2x), 1 µl of primers TGGTTCATGTCACATAAGGGAGTTC and

TTGCGTGCTCTAATAATGGCTTG (10 µM; salt free; Eurofins Genomics) each and 2 µl H_2O. The PCR program started with 3 min at 95 °C, followed by 34 cycles of 30 s at 95 °C, 30 s at 58 °C and 60 s at 72 °C and a final elongation for 5 min at 72 °C. Samples were analysed on a 1 % agarose gel (w/v), and a wild-type infected *A*. *graveolens* as well as a non-infected plant were chosen as positive and negative control.

4.3.4 Construction of an infectious full-length cDNA clone

Total nucleic acid was extracted using a silica-particle based method described by Menzel et al. (Menzel et al. 2002). Reverse transcription and PCRs were used in standard assays using 20 U/µl RevertAid Reverse Transcriptase (Thermo Fisher Scientific) and Phusion Flash High-Fidelity PCR Master Mix (Thermo Fisher Scientific). For the construction of a full-length cDNA clone, two fragments of 3,278 bp and 1,976 bp overlapping by 26 bp with each other and comprising approximately the 5'-half of the genome, were generated and assembled via Gibson Assembly into the vector pDIVA (empty vector map in figure 8.1, KX665539) (Gibson et al. 2009). In a second step, the 3'-half of the genome (excluding the poly(A) tail) was amplified in two fragments of 1,742 bp and 3,087 bp (also with an overlap of 26 bp) and assembled into the plasmid containing the 5`-half. Assembled products were transformed into *E*. *coli* NM522 and resulting plasmids were analysed by restriction enzyme digest (Hanahan 1983). Sequencing of the entire clone revealed a single nucleotide deletion causing a frameshift and leading to a premature stop codon in the ORF. Therefore, the first fragment was replaced by a newly amplified fragment from infected plant material. Sequencing revealed a correct fragment and the resulting clones were transformed into *R*. *radiobacter* GV2260 for subsequent infiltration into two different celery cultivars.(Grimsley et al. 1986). A plasmid map is illustrated in figure 8.2 (right).

4.3.5 Aphid transmission, sequence analysis and phylogeny

In the aphid transmission assay, the insects were allowed to feed on an infected celery plant for five to ten minutes to acquire the virus. Afterwards, ten aphids each were transferred to six healthy celery plantlets.

Sequence data were analysed using Geneious 10 and alignments as well as phylogenetic analyses were done with MEGA6 (Tamura et al. 2013). For the MUSCLE alignment, default parameters were chosen (DNA sequences: gap opening: -400, gap

extension: 0; protein sequences: gap opening: -2.9, gap extension: 0) (Edgar 2004). The phylogenetic tree is based on nucleotide sequences of the CP gene of seven CeMV isolates, eight other potyviruses and blackberry virus Y (*Brambyvirus*) as outgroup.

Tree construction was performed using the Neighbor-joining method (Bootstrap: 1000 replications, gaps/missing data treatment: complete deletion) (Felsenstein 1985; Saitou, Nei 1987). For calculation of percent identities, sequences of CeMV-Que and other isolates and viruses, respectively, were aligned and identity values displayed in BioEdit 7.2.5 (Hall 1999).

4.4 Results

In the host range test (Tab. 4.2), CeMV infected celery species and other members of the *Apiaceae* like *Daucus carota* and *Petroselinum crispum* but failed to infect any tested plants of the *Solanaceae* and *Amaranthaceae*. The symptoms occurred as deformation, yellowing and vein-clearing of the leaves.

Table 4.2: Host range of CeMV.

Plant species	**Infection**	**Plant species**	**Infection**
***Apiaceae/Apium* sp.**		***Apiaceae*-Other species**	
A. graveolens var. *rapaceum* 'Mars'	+	*Ammi majus*	+
A. graveolens var. *rapaceum* 'Ibis'	+	*Coriandrum sativum* 'Thüringer'	-
A. graveolens var. *rapaceum* 'Giant Prague'	+	*Daucus carota* L. 'Rote Riesen 2'	+
A. graveolens var. *rapaceum* 'Monarch'	+		
A. graveolens var. *rapaceum* 'Prinz'	+	***Amaranthaceae***	
A. graveolens var. dulce 'Gigante Dorato'	+	*Chenopodium amaranticolor*	-
A. graveolens var. *dulce* 'Tall Utah 52/70'	+	*Chenopodium foliosum*	-
A. graveolens var. *dulce* 'Sedano d'Elne'	+	*Chenopodium nubrum*	-
A. graveolens var. *dulce* ‚Bleich-Stangensellerie'	+	*Chenopodium quinoa*	-
A .graveolens var. dulce 'Goldener Riesensellerie'	+		
A. graveolens var. *secalinum* 'Gewone Snij'	+	***Solanaceae***	-
A. graveolens var. *secalinum* ‚Schnittsellerie'	+	*N. benthamiana*	-
A. graveolens var. *secalinum* ‚Krause Schnittsellerie'	+	*N. debneyi*	-
		N. hesperis	-

+: infection; -: no infection;

4.4.1 Sequence analysis and phylogeny

The complete genome of CeMV-Que (MF962880) is 10,000 nucleotides (nts) in length (excluding the poly(A) tail). It contains a large open reading frame (ORF; 9,543 nts) that encodes a polyprotein of 3,181 amino acids (aa) and is flanked by a 5'-untranslated region (UTR) of 186 nts and a 3'-UTR of 271 nts. Compared to CeMV-Cal with a genome size of 9,999 nts, CeMV-Que has one additional nucleotide in the 5'-UTR. Overall, the two isolates share a nucleotide sequence identity of 95 % and an amino acid sequence identity of 96 % in the polyprotein. The gene and protein sizes in the large ORF/polyprotein are the same and eight of the nine protease cleavage sites are identical, only the P1/HC-Pro site differs at position P3. For CeMV-Que this site is TIHY/S instead of TVHY/S. In comparisons of the 3'-terminal part of the CeMV-Que genome, comprising the NIb (partial), CP and 3'-UTR, with that of other CeMV isolates, identity values of 99 % were observed for a Dutch (Hol) isolate (AF203531.1) and 97 % for four Australian isolates (AF203532.1 [isolate WA]; AF203533.1 [isolate Vic], AF203534.1 [isolate SA] and AF203535.1 [isolate Old].

In the phylogenetic tree, based on nucleotide sequences of the coat protein gene, the CeMV isolates form a distinct cluster located close to *Apium virus Y* and *Carrot virus Y* as reported before (Fig. 4.1) (Xu et al. 2011b). The sequence of CeMV-Aschersleben is not included in the trees and alignments due to two putative sequencing errors causing a frameshift in the CP over a short period (amino acids 27 to 53) (Moran et al. 2002). In spite of the low genetic variability among the sequenced members of CeMV, the tree appears to provide evidence of a geographically-associated sub-clustering for the isolates from Australia, USA and Europe.

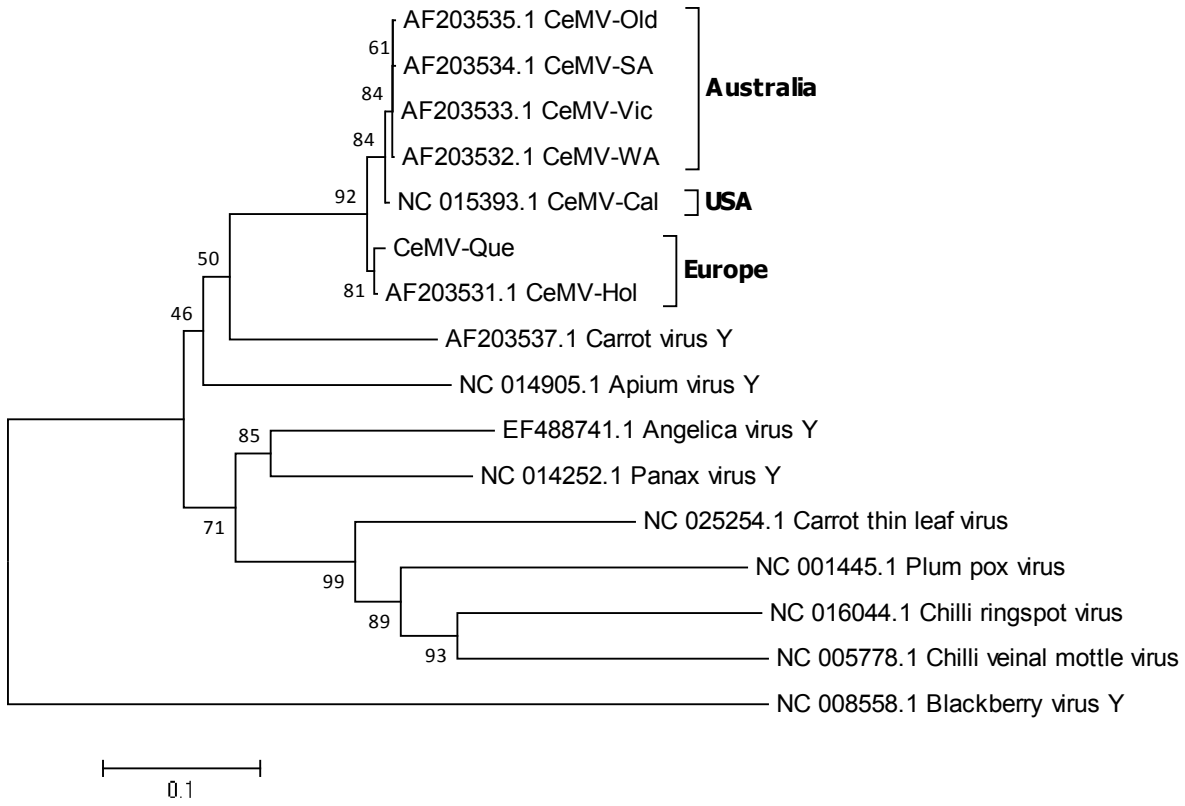

Fig. 4.1: Phylogenetic tree based on the coat protein-encoding nucleotide sequences of seven CeMV isolates, eight other potyviruses and blackberry virus Y (*Brambyvirus*) as the outgroup sequence. Alignment was performed by the Muscle alignment tool (Gap open: -400, gap extension: 0) (Edgar 2004) and tree construction using the Neighbor-joining method (Bootstrap: 1000 replications, gaps/missing data treatment: complete deletion) (Saitou, Nei 1987; Felsenstein 1985) in MEGA6 (Tamura et al. 2013). The numbers in front of the dividing nodes show the percentage bootstrap support. The scale bar shows the substitutions per site.

In alignments of CP nucleotide sequences, differences between the 'European' isolates and the others were found (Fig. 4.2, asterisks). In total, 13 out of 834 differing nucleotides were specific for CeMV-Que and CeMV-Hol, two of which cause an amino acid exchange. In spite of the high similarity between the amino acid sequences of all isolates, it is remarkable that CeMV-Que, CeMV-Aschersleben (not shown in the alignments) and CeMV-Hol have a NAG motif whereas a DAG is found in all the other isolates (except CeMV-SA: DAC). The aphid transmission assay for CeMV-Que was successful with four out of six plants being infected. In the 3'-terminal part of the NIb (nts 919-1554/aa 307-518) 22 nucleotide exchanges along with three amino acid substitutions are specific for the 'European' isolates (data not shown). Another difference between CeMV-Que and CeMV-Cal was found in the PIPO cistron, causing a premature stop codon in CeMV-Que resulting in an 18 nts shorter coding sequence and a six amino acid shorter protein (Fig. 4.2, asterisks).

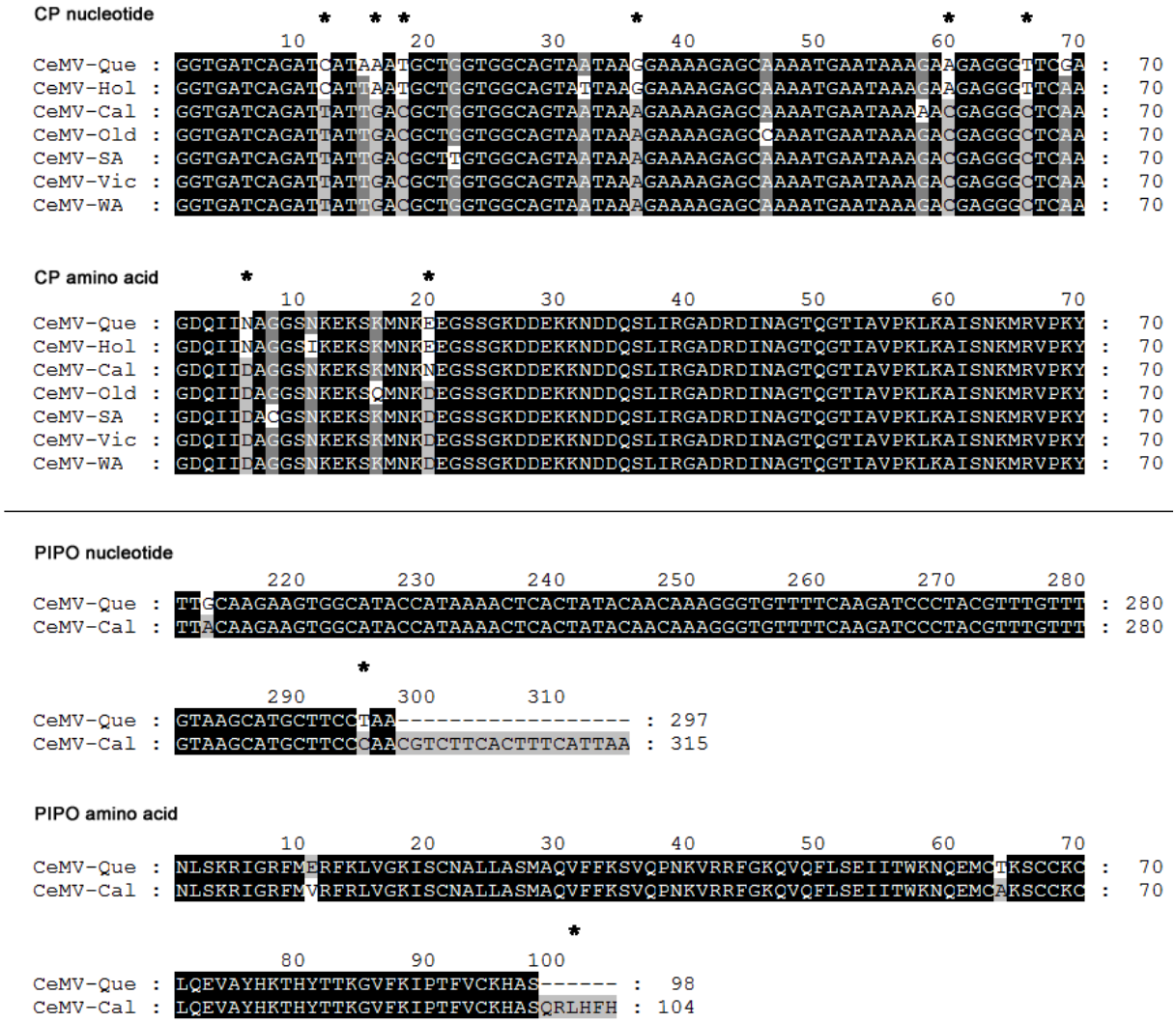

Fig. 4.2: Nucleotide and amino acid sequence alignments of a part of the CP and PIPO cistrons of CeMV-Quedlinburg (Que) and CeMV-California (Cal). Numbers above and on the right side of the alignment mark the nucleotide/amino acid position. Black shaded areas are identical and grey or white positions are of different identity. Positions mentioned in the text are marked with an asterisk (*) and gaps with a dash (-).

4.4.2 The infectivity of the full-length cDNA clone in *A. graveolens*

The infectivity of putative full-length clones was tested by infiltrating the *A. graveolens* cultivars ‘Mars’ and ‘Gewone Snij’ with *R. radiobacter*. At 14 days after mechanical inoculation, mild symptoms were observed for the wild-type virus. The plants infiltrated with the full-length cDNA clone showed first symptoms after 25 days (Fig. 4.3) resembling those of the wild-type virus.

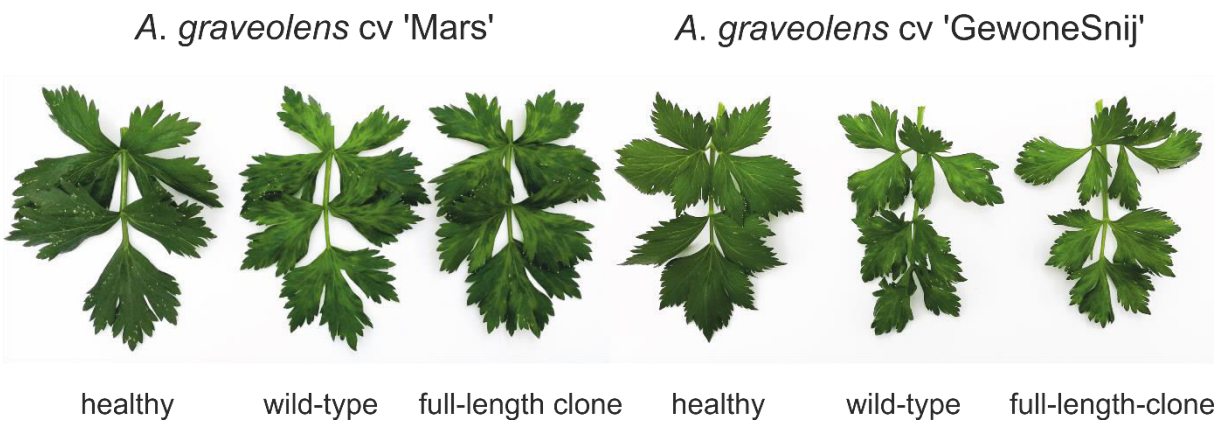

Fig. 4.3: Symptom development of infected *Apium graveolens* cvs 'Mars' and 'Gewone Snij' with celery mosaic virus Quedlinburg (DSMZ PV-1003) wild-type and full length clone compared to healthy controls 50 dpi.

The infection rates varied between four to five out of five plants and the symptom development of the full-length cDNA clone was delayed compared to the wild-type virus. The symptoms occurred mostly about ten days later. CeMV-Que infection was verified by RT-PCR followed by subsequent sequencing of the fragment.

4.5 Discussion

Celery mosaic virus has spread worldwide and has been described and characterized in different countries since the 1930s (Xu et al. 2011b). With regard to host plant spectra, there are reports of numerous isolates on the infectivity of various plants. In summary, it seems that the isolates can be divided into two categories. Either they infect only *Apiaceae* or *Apiaceae* as well as species from the *Amaranthaceae* (*Chenopodium* sp.) (Bos et al. 1989; Khoshkhatti et al. 2011). Additionally, there is a report of an Egyptian isolate which can infect *Solanaceae* (e.g. *Capsicum* sp. and *Nicotiana* sp.) in addition to the two families mentioned (Amal et al. 2012). CeMV-Que seems to be a member of the group which can infect *Apiaceae* only.

In the phylogenetic tree (Fig. 4.2) of CP nucleotide sequences the CeMV isolates seem to form clusters depending on the geographic origin (Europe, USA, Australia), which supports previous findings (Moran et al. 2002). To date, nothing is known about the contribution to such a differentiation by environmental influences or mutation of symptom determinants in CeMV isolates at the molecular level. Since so far only one complete sequence of a Californian CeMV is available (NC_015393.1), reliable

comparisons between all genes and proteins are not yet possible, but there are first indications of possible interesting facts. The so called “European” CeMVs Aschersleben, Hol and Que show a NAG motif instead of the mostly occurring DAG in the coat protein. The DAG motif is highly conserved in the genus *Potyvirus* and is involved in aphid-transmission by interacting with the HC-Pro (Atreya et al. 1990; Atreya, Pirone 1993). Although an NAG motif is infrequently found, there is some controversy about the significance of this motif for aphid transmissibility. Whereas TVMV, bean yellow mosaic virus (BYMV), ranunculus leaf distortion virus (RanLDV) and Ranunculus mild mosaic virus (RanMMV) possessing this type of motif were transmissible by aphids (Atreya et al. 1995; Wylie et al. 2002; Turina et al. 2006) no aphid transmission was observed for the *Plum pox virus* isolates C and NL (Kamenova et al. 2002). CeMV-Que is transmissible by aphids and the question arises whether this mutation developed as a result of changing vector species in this area or just by chance. In further sequence comparisons there are some positions only to be found in the “European” isolates and the P3N-PIPO of CeMV-Que is six amino acids shorter than CeMV-Cal. Taken together, all these different characteristics of the genomes and polyproteins could be a hint to a possible adaption of the individual isolates to the host and vector composition. P3N-PIPO is involved in viral movement guiding viral ribonucleoproteins in combination with the cylindrical inclusion protein to the plasmodesmata (Wei et al. 2010b). A direct interaction with a host protein, the hydrophilic plasma membrane-associated cation binding protein PCaP1, could be demonstrated for *Turnip mosaic virus* (TuMV) (Vijayapalani et al. 2012). Additionally, it is known that varying P3N-PIPO lengths within a species are not uncommon and in case of TuMV isolates affect their fitness in different host plants (Hillung et al. 2013). To address the question whether the differences in the genomes of CeMV isolates could be traced back to changing environmental conditions, more sequences of “European” and other CeMV samples are needed along with knowledge about the exact origin and host range. With the infectious full-length cDNA clone of CeMV-Que it would be possible to identify putative sequence parts being responsible for the host range of the virus by introducing mutations into the existing clone followed by infectivity assays. In studies from Salvador *et al*. it was shown that host-specificity determinants are located in P1, P3, 6k1 and the N-terminus of the CP. They used chimeric GFP labelled PPV strains as well as specific mutations combined with infection experiments in *N*. *clevelandii* and peach (Salvador et al. 2008a). Additionally, it is known, that

symptom determinants are located in the region of the P3 C-terminus to 6K1 (Sáenz et al. 2000). These findings indicate that differences in host-ranges of CeMV isolates could possibly be traced back to mutations in these regions.

The delayed symptom development of the full-length cDNA clone in the infiltrated plants can possibly be explained by inefficient infiltration of the bacterial suspension according to the natural stable character of celery leaves. In a mechanical inoculation the initial virus titer is probably much higher and the virus particle itself is transferred to the plant. In case of the full-length clone, the T-DNA containing the virus sequence is transferred into the nucleus, which, first of all, has to be transcribed and exported into the cytoplasm where it is then translated and able to replicate and to form virions (Gelvin 2003). Nevertheless, the availability of an infectious full-length clone of CeMV now offers the opportunity to study putative hosts and symptom determining sequence sections of this virus.

5 General Discussion

In this thesis, two celery-infecting viruses were characterized biologically and molecularly. This involved, on the one hand, the unassigned celery latent virus, which has not yet been investigated in molecular terms but considered to be a member of the the *Potyviridae* and, on the other hand, a German isolate of the *Celery mosaic virus* (MF962880), which belongs to the genus *Potyvirus* in this family (Brandes, Luisoni 1966; Bos et al. 1978; Xu et al. 2011b). For both viruses it was possible to obtain the complete genome sequence and to construct an infectious full-length cDNA clone by using Gibson Assembly (for CeMV) or a combined strategy based on Gibson Assembly and restriction-based ligations (for CeLV) (Gibson et al. 2009).

The *Celery mosaic virus* isolate from Quedlinburg described in the chapter before is closely related to the other ones from Australia, California and the Netherlands, and could thus be confirmed as an isolate of the species (Adams et al. 2005b). By determining the complete genome sequence, CeMV Quedlinburg is the second fully sequenced virus of the species besides CeMV California (NC_015393.1) (Xu et al. 2011b). A phylogenetic analysis of the CP nucleotide sequences showed a division of the isolates according to their origin: Australia, USA and Europe. This fact was previously described by Moran et al (Moran et al. 2002). This kind of division could be observed for other *Potyvirus* species. In case of *Zucchini tigre mosaic virus* there was an Asian, American as well as an Indian ocean cluster and the *Papaya ringspot virus* isolates divided to Asian or American clusters (Romay et al. 2014). Analyses of TuMV isolates also revealed a potential correlation between evolutionary changes and geographic origin, associated with adaptation to new host plants for example (Ohshima et al. 2002). Investigations on genetic diversity of *Tobacco mild green mosaic virus* (*Tobamovirus*) isolates in wild *N. glauca* plants also revealed variations among populations from different origins (Fraile et al. 1996). This indicates, that transmission by animal vectors is not the necessarily needed for a genetic divergence.

More detailed analyses of the partial NIb, CP and 3'-UTR sequences at the nucleotide and amino acid level revealed mutations, some of which are specific to European isolates. The most striking example is the DAG to NAG mutation in the CP protein. DAG is a highly conserved motif involved in the transmission by aphids and changes or non-existence of this motif often lead to loss of transmissibility (Atreya et al. 1990;

Atreya et al. 1995; Wylie et al. 2002; Turina et al. 2006). The NAG variant has been found in several viruses and the aphid transmissibility, also for CeMV-Que, has been confirmed (Atreya et al. 1995; Wylie et al. 2002; Turina et al. 2006). The reason why CeMV-Hol and CeMV-Que show an NAG motif is not yet known. It can only be assumed that various environmental influences, such as varying aphid species or the different composition of host plants of the geographical origins have led to these changes. There is for example the theory about genetic bottlenecks in different stages of the viral infection cycle, like invasion of new hosts, short- and long-distance movement as well as transmission by vectors. Within a virus population, sequence variations emerge and a certain number is transported through the vascular system in the systemic leaves. Not all members of the population are equally successful and viruses, which are highly similar, can exclude each other from the same cell resulting in a segregation within a leaf. In addition, it was shown that one aphid transmitted 0.5 to 3.2 viral genomes averagely (Gutiérrez et al. 2012). It could be possible, that the "NAG variant" of CeMV-Que had some kind of advantage like a slightly increased transmission rate by aphids, which led to the stabilization of this mutation in the population.

Another indication is the P3N-PIPO shortened by six amino acids compared to CeMV California. P3N-PIPO was shown to interact directly with the host´s hydrophilic plasma membrane-associated cation binding protein PCaP1 (Vijayapalani et al. 2012). Varying P3N-PIPO lengths were also observed in case of TuMV isolates and could be shown to play a role in the adaption to and fitness in different host plants (Hillung et al. 2013). These assumptions, however, would require much more sequence data with precise details of their origin and host-range spectra. In the literature, isolates differ in their host plant spectrum in that they either only affect *Apiaceae* or *Apiaceae* and *Amaranthaceae* (Brandes, Luisoni 1966; Bos et al. 1989; Khoshkhatti et al. 2011). An isolate from Egypt also infects *Solanaceae* species (Amal et al. 2012). Nothing is known about the molecular background, because of the isolates which are deposited in the GeneBank, no data about host plants are available except for CeMV-Hol, which also seems to infect only *Apiaceae* (Bos et al. 1989). It would be very interesting to obtain the complete sequences from the isolates with a wider host range but also CeMV-Hol to compare them. This would also provide more information about the individual proteins and not only about the CP. Today it is known that several proteins (also in combination) can have an influence on the infectivity and symptom expression

in different host plants. The pathogeny determinants are located on several positions within the PPV polyprotein are for example P1, HC-Pro, P3, 6K1, NIb and the N-terminus of CP. In experiments in which chimeras were constructed from two different PPV strains, the symptomatology determinants could be limited to the range from P3 C-terminus to 6K1 (Sáenz et al. 2000). After passages with and without shifting host plants the virus populations were screened for mutations. Experiments with PPV on peaches and peas showed that the infectivity increased after two passages after a change from peach to pea. For example, a mutation in the NIb that led to an amino acid exchange which could be an indication of potential adaptation to the new host. Even after 40 passages on pea, PPV was able to infect the original host (Wallis et al. 2007). Some PPV isolates lost this ability of infecting the woody host after long-term propagation in herbaceous hosts. Construction of hybrid viruses comprising sequences of two PPV strains [D (infects peach) and R (unable to infect peach)] led to the speculation, that P1, P3, 6K1 and the N-terminus of CP are involved (Salvador et al. 2008a). Similar experiments were performed by exchanging P1 between PPV-D and TVMV. PPV carrying P1 of TVMV was still able to infect *Nicotiana* sp. but unable to infect *Prunus* sp., indicating the influence of P1 of the host spectrum (Salvador et al. 2008b). All of these results seem to be host-specific so that a generalization is difficult. For CeMV, individual experiments for determining the involved sequence segments would have to be carried out. We could show the successful infection of celery and celeriac species with the full-length clone via *Rhizobium* mediated infiltration. This tool could be used to exchange entire regions or proteins between two or more CeMV isolates or individual motifs and amino acids with following infection tests. For example, this could be used to test which protein or motif is responsible for limiting certain isolates on plants of *Apiaceae*. In combination with detailed information on the origin and spread of the isolates, the possible evolutionary development of *Celery mosaic virus* could possibly be elucidated. It would be interesting to know, if the isolates gained or lost the wider host-range infectivity over time and during migration.

All tested *Apium graveolens* species in this study were susceptible to CeMV-Que (Tab. 4.2). This isolate was collected from a symptomatic celery plant in Quedlinburg but there are no reports of high incidences in Germany. But the virus occurs worldwide and caused massive crop losses for example in California and Australia (Severin, Freitag 1938; Latham, Jones 2003). In these two countries, if a severe infestation with CeMV is established, a three month celery free period is carried out, which functions

as a suitable counter measure. Alternative hosts of celery such as dill, fennel and coriander are also removed. This strategy works well for about two years but after that, a slight infestation could be observed again. However, these measures are only of short duration and are not a long-term solution. The cultivated celery varieties are highly susceptible to CeMV (e. g. cv Tendercrisp) and resistant varieties are not yet known (Latham, Jones 2003). Celery free periods are also not successful everywhere. For example, in England CeMV infects many alternative wild hosts, such as wild hemlock. These plants serve as a reservoir for the virus, making it difficult to control (Ruiz et al. 2001). There are reports of resistance against CeMV in a feral celery variety, a locus named *cmv* (D'Antonio et al. 2001). Molecular markers have also been developed to facilitate selection in the breeding of resistant varieties (Ruiz et al. 2001). A faster technology for the generation of resistances against potyviruses is the CRISPR/Cas9 method. The successful application could be demonstrated, for example, on cucumbers where the *eIF4E* gene served as a target. Plants of the homozygous T3 line were transgene-free and resistant to CVYV, *Zucchini yellow mosaic virus* (ZYMV) and PRSV (Chandrasekaran et al. 2016). Similar results were obtained in experiments on knock-out of the *eIF4E* analogue *eIF(iso)4E* and resistance to TuMV in *Arabidopsis thaliana* (Pyott et al. 2016). With the help of this technology, it might be possible to quickly integrate resistance into the most widely cultivated celery varieties which could help growers in CeMV affected areas.

In contrast to CeMV the celery latent virus is symptomless. CeLV has probably no direct economic significance. In the literature there are controversial discussions about symptoms and possible effects of mixed infections. CeLV was first discovered in a stunted plant with slightly yellowed veins. Brandes and Luisoni could not reproduce these symptoms in experiments with *Apium* sp., they just discovered faint mosaic in only few plants. Their suspicion of a mixed infection of the original plant could not be verified (Brandes, Luisoni 1966). Bos et al. mentioned results from previous studies of Verhoyen et al. in which CeLV led to yield reductions of about 33 % to 38 % in different celery species. Bos et al never observed symptoms in CeLV infected celery and a mixed infection with CeMV did not lead to increased symptoms. They did not collect data about possible yield reductions and so far the economic significance of CeLV remains unclear (Bos et al. 1978). On the molecular level, little was known about celery latent virus until today and initial assessments based on particle length and morphology revealed similarities to the *Potyviridae* despite the absence of cylindrical

inclusion bodies which are typical for potyvirids (Brandes, Luisoni 1966; Bos et al. 1978). In this work it was possible to determine the complete genome sequence of an Italian isolate (Ag097). Numerous bioinformatic analyses have shown that CeLV is an unusual putative member of a putative new genus in the *Potyviridae* family. These special characteristics made the taxonomic classification more difficult and there are arguments for and against a membership within this family. Characteristics that speak against classification as a potyvirid are on the one hand a very short 5'-UTR of only 13 nucleotides, weak correspondence to known cistrons and proteins (motifs), the absence of a 3'-poly(A) tail as well as cylindrical inclusion bodies and on the other hand the presence of a functional N-terminal localised signal peptide, which leads proteins to or into the endoplasmic reticulum. To our knowledge, the last three characteristics are unique for CeLV and not described for any other potyvirid (Wylie et al. 2017). We are convinced that the arguments for a membership of the family predominate, because there are certain similarities. The CeLV genome codes for a large ORF, from which a polyprotein is translated. This consists of several proteins that have to be cleaved into their individual functional units. It could be confirmed that a silencing suppressor is located in the N-terminal region of the CeLV polyprotein, a P3N-PIPO is generated in the putative P3 cistron by a ribosomal frameshift at the conserved motif $G_{1-2}A_{6-7}$ and the order of the predicted known motifs for a CI, NIa-Pro, NIb and CP resemble those of other potyvirids (Wylie et al. 2017). These facts are supported by the position of CeLV in the phylogenetic tree, where it clusters near the branches of the genera *Bymovirus*, *Macluravirus* and bevemovirus. The calculation of the percentage identities of the polyprotein, NIb and CP from CeLV to the type members of each genus clearly indicate the presence of a new genus with the proposed name celavirus. However, the determination of the vector, exact protease cleavage sites and the number, identity as well as the function of the N-terminal proteins are still not known yet.

The *Potyviridae* contains currently eight accepted genera and 195 species (two unassigned) (Wylie et al. 2017). The *Potyvirus* group is the largest one with 160 members and thought to be evolved from the rymoviruses (Gibbs, Ohshima 2010). The other genera of the family are not uniform, but on the contrary very different in terms of deviations from the typical potyviral genome organization. The discovery of new genomic elements is not unusual which is illustrated by the following examples. The bymoviruses, transmitted by root-infecting organisms of the *Plasmodiophorales*,

are the only genus with a bipartite genome. Two proteins are located on RNA2. P1 shows similarities to the C-terminal part of the HC-Pro of potyviruses and is thought to be a cysteine protease and P2, of which the functions are still unknown except for a possible involvement in vector transmission (Kashiwazaki et al. 1990; Kashiwazaki et al. 1991). Within this genus, *Oat necrotic mottle virus* (OMV) comprises an uncommonly short 5'-UTR of 77 nucleotides and a deleterious P2 indicating that members of one genus do not strictly follow the common patterns (Zheng et al. 2002). Latest phylogenetic analyses propose subgrouping the bymoviruses into two groups: 1 comprises BaYMV, *Wheat yellow mosaic virus* (WYMV) and OMV, 2: RNMV and BaMMV (Wagh et al. 2016). And this is not the only example of genomic variation. In the case of ipomoviruses, the N-terminal area varies with the presence of both P1 and HC-Pro (SPMMV: P1 as silencing suppressor, TMMoV: silencing suppressor not studied), P1a and P1b (CVYV and SqVYV: P1b as silencing suppressor) and P1 without HC-Pro or P1b (CBSV and UCBSV) (Abraham et al. 2012; Dombrovsky et al. 2014). The macluraviruses lack a P1, their genome directly starts with a truncated HC-Pro (silencing suppression not studied) but are still transmissible by aphids and seen as an evolutionary link between bymoviruses and other potyvirids (Kondo, Fujita 2012; Zhang et al. 2016). Two additional genera are discussed by the ICTV at the moment. One of them is named bevemovirus with bellflower veinal mottle virus (BVMoV) and the other one roymovirus with rose yellow mosaic virus (RYMV) as the type member (clusters near the branches of poaceviruses and tritimoviruses) (Mollov et al. 2013; Seo et al. 2017b). Another two viruses that may represent new genera have been published but have not yet been discussed by the ICTV and seem not be closely related to CeLV: longan witches' broom-associated virus, which clusters near the branch of RYMV and common reed chlorotic stripe virus, placed between the genera *Brambyvirus* and *Rymovirus* (not included in the phylogenetic tree) (Seo et al. 2017a; Yuan et al. 2017). Still much new members are identified and thus new sequence information is generated. It would be important to find a second virus that resembles CeLV and belongs to the same genus. BVMoV has the same genomic organization as macluraviruses (P1 protease is absent) but phylogenetic and sequence analyses consider the membership of a putative new genus and in addition support the assumption of an evolutionary link between the bymoviruses and others. In the phylogenetic tree CeLV is most closely associated to the branches of BVMoV, macluraviruses and bymoviruses, indicating a putative common ancestor. However,

the evolutionary backgrounds are still unknown due to significant sequence variations of CeLV compared to all other members. It is the largest fully sequenced member of the family to date which is a contrast to the macluraviruses, having the smallest genome within the family. There is a speculative theory that the bymoviruses are the most ancient potyvirids (Kondo, Fujita 2012). A further indication of this can be found in connection with the considerations about the evolution of HC-Pro. (Valli et al. 2017). It is mentioned that the bymoviral cysteine protease could be an ancestor of a first HC-Pro like protein as for example the truncated version in the macluraviruses. These findings lead to a highly speculative theory concerning CeLV. Based on all information and the premise that CeLV is accepted as a member of the family, it could be seen as potential distant ancestor of bevemoviruses, bymoviruses as well as macluraviruses and thus of all potyvirids. This hypothesis is on the one hand based on the position of CeLV in the phylogenetic tree and on the other hand based on the uncommon composition of the N-terminus with its signal peptide and non-identifiable proteins with high sequence variations compared to every other potyvirid as well as the absence of a poly(A) tail. However, intense studies are needed to confirm CeLV's position in the evolutionary process, especially the origin and loss of the signal peptide as well as the emergence of the longer 5'-UTR and poly(A) tail of potyvirids have to be investigated.

To date it was not possible to identify the N-terminal located proteins and features completely. Their investigation is essential for the validation of CeLV's evolutionary position. It could just be speculated, that one or two proteases are likely or that this part of the genome is cleaved by the NIa, too. The existence of a completely new genomic element which is guided to or into the endoplasmic reticulum by the signal peptide is also conceivable. N-terminal located signal peptides are barely reported for plant viruses. Tospoviruses like bean necrotic mosaic virus and TSWV encode putative signal peptides in the glycoproteins (Kormelink et al. 1992; Oliveira et al. 2012). They lead glycoproteins to and from the ER to the Golgi (Ribeiro et al. 2008; Chen et al. 2012). Recent studies of *Tomato ringspot nepovirus* revealed the presence of a signal peptidase cleavage site in the C-terminal region of the NTB-VPg protein, an integral membrane replication protein, downstream of a amphipathic helix and hydrophobic domain (Wei et al. 2016). The VPg domain, harbouring a putative glycosylation site N-X-T, was found to be translocated into the ER lumen and is later on glycosylated. In the CeLV polyprotein, a possible N-glycosylation site, following the N-X-S/T pattern (N-L-T, positions 51-53), can be found. Potyvirids usually target the ER with their 6K2

protein, which is verified as a membrane anchor. The replication complex is guided to the chloroplasts in ER-derived vesicles where replication takes place (Schaad et al. 1997a; Wei et al. 2010a). It could be speculated, that CeLV follows a divergent replication strategy by using an N-terminal located signal peptide, applying the function of an ER membrane anchor. Analyses with the TMpred server (Hofmann 1993) revealed a membrane spanning helix comprising the amino acids five to 21 of the CeLV signal peptide. Additionally, it could be assumed that another replication strategy would need an adapted larger NIb (721 amino acids). This theory has not been investigated in this study and further experiments are to be made to get an insight into the biology of CeLV.

The first attempt to identify possible protein functions was the localization of a putative silencing suppressor. For this, the method of a transient local silencing assay was performed as described previously (Varrelmann et al. 2007). With this approach it was possible to verify a silencing active region within the first 703 amino acids. Due to limitations of the method, a further demarcation was unfeasible. For future experiments, the question of how many proteins of which size are located in the N-terminus should be clarified first. CeLV proteins can for example be produced using cell-free coupled *in vitro* translation systems. This requires clones with parts of the polyprotein of different length from the N-terminus. Emerging proteins need to be labelled and detected afterwards by either SDS gel electrophoresis followed by a laser-based scan or by a western blot. An alternative strategy is to create constructs of different lengths from the N-terminal region, which are bacterially expressed and can be purified and detected with the help of tags (e.g. Strep-Tag). Depending on the position of viral proteases in combination with the placement of the tag, it could be possible to determine whether a protease is active and how many proteins with the determination of their approximate sizes are present. For the exact size determination and identification of a protein, it is necessary to purify it with subsequent protein sequencing. Similar methods have already proven the protease activity of potyviral proteins like P1 (Verchot et al. 1991). The cleavage sites and accordingly sizes of all other proteins, especially the putative large NIb, have to be elucidated.

This work revealed the first complete genome sequence of celery latent virus and a German *Celery mosaic virus* isolate from Quedlinburg. CeMV-Que is a typical *Potyvirus* and the taxonomic classification into the CeMV clade led to speculations

about evolutionary changes in the genome sequence during migration and adaption to new environments. The other celery-infecting virus, CeLV, represents an unusual member of a putative new genus within the *Potyviridae* with a yet unique genome organization which has not been described for potyvirids before. Due to the successful construction of an infectious full-length cDNA clone for both viruses, it is now possible to perform further investigations of the protein functions and to discover the still unknown features of CeLV.

6 References

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7 Abbreviations

Δ	delta
%	percent
35S P	CaMV 35S promoter
aa	amino acids
A. g.	*Apium graveolens*
A. graveolens	*Apium graveolens*
AGO	Argonaute
as	antisense
b	base(s)
BI	back inoculation
Blast (n, x, p)	Basic Local Alignment Search Tool (nucleotides, translated nucleotide query, protein)
bp	base pair(s)
°C	degree Celsius
C	cytosine
C-terminus/-terminal	carboxyl-terminus/-terminal
C.	*Chenopodium*
C. quinoa	*Chenopodium quinoa*
Cal	California
cDNA	complementary DNA
CI	cylindrical inclusion
CLSM	confocal laser scanning microscopy
CP	coat protein
C-terminus/terminal	carboxyl terminus/terminal
cv(s)	cultivar(s)
Da	dalton
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
dGTP	deoxyguanosine triphosphate
DIECA	diethyldithiocarbamate sodium
DNA	deoxyribonucleic acid
dNTP(s)	deoxynucleotide(s)

dpi	days post inoculation
ds	double-stranded
dsDNA/RNA	double-stranded DNA/RNA
dsRed	*Discosoma* sp. red fluorescent protein
DSMZ	Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures
D strain	Dideron strain
dTTP	deoxythymidine triphosphate
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
eIF4E	eukaryotic translation initiation factor 4E
ER	endoplasmic reticulum
ERES	endoplasmic reticulum exit sites
et al.	*et alii*
Fig.	figure
g	gram
G	guanosine
GFP	green fluorescent protein
h	hour(s)
HC-Pro	helper-component protease
HDV	*Hepatitis delta virus*
HDVagrz	*Hepatitis delta virus* antigenomic “core” ribozyme
Hol	Holland
ICTV	International Committee On Taxonomy Of Viruses
IRES	internal ribosome entry site
ISEM	immunosorbent electron microscopy
kb	kilobase(s)
kbp	kilobase pair(s)
kDa	kilodalton(s)
kV	kilovolt
LB	lysogeny broth
M	molar
µg	microgram
mg	milligram

µl	microliter
ml	milliliter
µM	micromolar
µl-1	per microliter
MES	2-(*N*-morpholino)ethanesulfonic acid
min	minute(s)
miRNA	microRNA
ml	milliliter
mm	millimeter
mM	millimolar
MP	movement protein
mRFP	monomeric Red Fluorescent Protein
mRNA	messenger RNA
M strain	Markus strain
MW	molecular weight
N.	*Nicotiana*
N. *benthamiana*	*Nicotiana benthamiana*
N-terminus/-terminal	amino-terminus/-terminal
Na-DIECA	sodium diethyldithiocarbamate
NAT	aphid non-transmissible
NCBI	National Center for Biotechnology Information
NI	nuclear inclusion
NLS	nuclear localization signal
nm	nanometer
nosT	nopaline synthase terminator
nptIII	kanamycin resistance
nt(s)	nucleotide(s)
NTP(s)	nucleoside-triphosphate(s)
NTPase	nucleoside-triphosphatase
OD	optical density
ORF	open reading frame
ori	origin of replication
pA35S/ 35S pA	polyadenylation signal of CaMV
PABP	poly(A)-binding protein

PAGE	polyacrylamide gel electrophoresis
PCaP1	plasma membrane-associated cation-binding protein 1
PCR	polymerase chain reaction
pH	potential hydrogen
Phyre	Protein Homology/118nalogy Recognition Engine V 2.0
PIPO	Pretty Interesting *Potyviridae* ORF
PISPO	Pretty Interesting sweet potato *Potyvirus* ORF
Pro	protease
PTGS	post-transcriptional gene silencing
Que	Quedlinburg
RACE	rapid Amplification of cDNA Ends
RdRp	RNA-dependent RNA polymerase
RFLP	restriction fragment length polymorphism
RISC	RNA-induced silencing complexes
RNA	ribonucleic acid
RNAi	RNA interference
rpm	rounds per minute
R. radiobacter	*Rhizobium radiobacter*
R strain	Rankovic isolate
RT-(Buffer,PCR)	reverse transcription/reverse transcriptase PCR
s	second(s)/sense
SA	South Australia
SDS	sodium dodecyl sulfate
siRNA	small interfering RNA
SMART	Simple Modular Architecture Research Tool
smRS-GFP	soluble-modified red-shifted GFP
SOC	Super Optimal broth with Catabolite repression
SRP	signal recognition particle
sp.	species
SP	signal peptide
spp.	Species pluralis
ss	single-stranded
ssDNA/RNA	single-stranded DNA/RNA
(+)ssRNA	positive orientated single-stranded RNA

T	thymine
TEM	transmission electron microscopy
trfA	plasmid replication initiator protein
tRNA	transfer RNA
U	unit(s)
UTR	untranslated region
UV	ultraviolet
V	volt
var	variety
Vic	Victoria
VIGS	virus-induced gene silencing
VPg	viral genome-linked protein
VRC	Viral replication complex
WA	Western Australia
WT	wild-type

Viruses

AgMV	*Agropyron mosaic virus*
AMV	*Alfalfa mosaic virus*
AnVY	*Angelica virus Y*
ApVY	*Apium virus Y*
ArLV	*Artichoke latent virus*
ArMV	*Arracacha mottle virus*
BaMMV	*Barley mild mosaic virus*
BaYMV	*Barley yellow mosaic virus*
BCNMV	*Bean common mosaic necrosis virus*
BlVY	*Blackberry virus Y*
BlScV	*Blueberry scorch virus*
BMV	*Brome mosaic virus*
BNMV	*Bean necrotic mosaic virus*
BrSMV	*Brome streak mosaic virus*
BVMoV	bellflower veinal mottle virus
BYMV	*Bean yellow mosaic virus*
CalVA	*Caladenia virus A*

CBSV	*Cassava brown streak virus*
CdMV	*Cardamom mosaic virus*
CeLV	celery latent virus
CeMV	*Celery mosaic virus*
CMV	*Cucumber mosaic virus*
CVYV	*Cucumber vein yellowing virus*
CYNMV	*Chinese yam necrotic mosaic virus*
DVY	*Daphne virus Y*
GVA	*Grapevine virus A*
HoMV	*Hordeum mosaic virus*
MacMV	*Maclura mosaic virus*
OMV	*Oat mosaic virus*
ONMV	*Oat necrotic mottle virus*
PanVY	*Panax virus Y*
PCV	*Peanut clump virus*
PPV	*Plum pox virus*
PRSV	*Papaya ringspot virus*
PSbMV	*Pea seed-borne mosaic virus*
PVY	*Potato virus Y*
RaLV	*Ranunculus latent virus*
RanLDV	*Ranunculus leaf distortion virus*
RanMMV	*Ranunculus mild mosaic virus*
RGMV	*Ryegrass mosaic virus*
RNMV	*Rice necrosis mosaic virus*
RYMV	rose yellow mosaic virus
SCSMV	sugarcane streak mosaic virus
SLRSV	*Strawberry latent ringspot virus*
SMV	*Soybean mosaic virus*
SPFMV	*Sweet potato feathery mottle virus*
SPMV	*Spartina mottle virus*
SPMMV	*Sweet potato mild mottle virus*
SqVYV	*Squash vein yellowing virus*
TBSV	*Tomato bushy stunt virus*
TEV	*Tobacco etch virus*

TMMoV	*Tomato mild mottle virus*
TMV	*Tobacco mosaic virus*
TOgMV	*Tall oat-grass mosaic virus*
ToRSV	*Tomato ringspot virus*
TRSV	*Tobacco ringspot virus*
TriMV	*Triticum mosaic virus*
TSWV	*Tomato spotted wilt virus*
TuMV	*Turnip mosaic virus*
TVMV	*Tobacco vein mottling virus*
TYLCV	*Tomato yellow leaf curl virus*
UCBSV	*Ugandan cassava brown streak virus*
WEqMV	*Wheat eqlid mosaic virus*
WSMV	*Wheat streak mosaic virus*
WYMV	*Wheat yellow mosaic virus*
YoGMV	*Yellow oat-grass mosaic virus*
ZTMV	*Zucchini tigre mosaic virus*
ZYMV	*Zucchini yellow mosaic virus*

Amino acids

A	Ala	Alanine
C	Cys	Cysteine
D	Asp	Aspartic acid
E	Glu	Glutamic acid
F	Phe	Phenylalanine
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
K	Lys	Lysine
L	Leu	Leucine
M	Met	Methionine
N	Asn	Asparagine
P	Pro	Proline
Q	Gln	Glutamine

R	Arg	Arginine
S	Ser	Serine
T	Thr	Threonine
V	Val	Valine
W	Trp	Tryptophan
Y	Tyr	Tyrosine

8 Addendum

8.1 Plants used for the host range tests of CeLV and CeMV

The following table lists all plant species and their provider.

Tab. 8.1: Origin of plant species used for the host range tests.

Plant species	Provider
Apiaceae*/Apium* sp.	
A. graveolens var. *dulce* 'Tall Utah 52/70'	Sperli
A. graveolens var. *dulce* 'Sedano d'Elne'	Flortis/Orvital
A. graveolens var. *dulce* ‚Bleich-Stangensellerie'	Dürr Samen
A. graveolens var. *rapaceum* 'Mars'	Sperli
A. graveolens var. *rapaceum* 'Ibis'	Dürr Samen
A. graveolens var. *rapaceum* 'Giant Prague'	Agrosel
A. graveolens var. *rapaceum* 'Monarch'	Hild
A. graveolens var. *rapaceum* 'Sedano rapa'	Flortis orto
A. graveolens var. *rapaceum* 'Prinz'	Kiepenkerl
A. graveolens var. *dulce* 'Gigante Dorato'	Franchi Sementi
A .graveolens var. *dulce* 'Goldener Riesensellerie'	Vilmorin Jardin
A. graveolens var. *secalinum* 'Gewone Snij'	Kiepenkerl
A. graveolens var. *dulce* 'Tall Utah 52/70'	Sperli
A. graveolens var. *dulce* 'Sedano d'Elne'	Flortis/Orvital
A. graveolens var. *dulce* ‚Bleich-Stangensellerie'	Dürr Samen
A. graveolens var. *secalinum* ‚Krause Schnittsellerie'	Rühlemann's Kräuter & Duftpflanzen
A. graveolens var. *secalinum* ‚Schnittsellerie'	Dürr Samen
***Apiaceae* -Other species**	
Coriandrum sativum 'Thüringer'	Quedlinburger
Daucus carota L. 'Rote Riesen 2'	Quedlinburger
Petroselinum crispum 'Gigante d'Italia'	Dürr Samen
Amaranthaceae	
Atriplex hortsensis	Kiepenkerl
Beta vulgaris subsp. *Vulgaris* 'Mangold Lucullus'	Frankonia
Chenopodium amaranticolor	Institute of Horticultural Production Systems, Sect. Phytomedicine
Chenopodium foliosum	Institute of Horticultural Production Systems, Sect. Phytomedicine
Chenopodium nubrum	Institute of Horticultural Production Systems, Sect. Phytomedicine
Chenopodium quinoa	Institute of Horticultural Production Systems, Sect. Phytomedicine

Tab. 8.1: Origin of plant species used for the host range tests.

Plant species	Provider
Spinacia oleracea 'Matador'	Kiepenkerl
Spinacia oleracea 'Monoppa'	Kiepenkerl
***Solanaceae/Nicotiana* sp.**	
N. benthamiana	Institute of Horticultural Production Systems, Sect. Phytomedicine
N. clevelandii	Institute of Horticultural Production Systems, Sect. Phytomedicine
N. debneyi	Institute of Horticultural Production Systems, Sect. Phytomedicine
N. edwardsonii	Institute of Horticultural Production Systems, Sect. Phytomedicine
N. hesperis	Institute of Horticultural Production Systems, Sect. Phytomedicine
N. occidentalis	Institute of Horticultural Production Systems, Sect. Phytomedicine
N. tabacum var. 'Samsun nn'	Institute of Horticultural Production Systems, Sect. Phytomedicine
N. tabacum var. 'Samsun NN'	Institute of Horticultural Production Systems, Sect. Phytomedicine
N. tabacum var. 'Xanthi'	Institute of Horticultural Production Systems, Sect. Phytomedicine
N. tabacum var. 'Xanthi nc'	Institute of Horticultural Production Systems, Sect. Phytomedicine

8.2 Accession numbers of sequences used for sequence and phylogenetic analyses

Table 8.2 displays all sequences used for the construction of the phylogenetic tree.

Tab. 8.2: Virus sequences used for the construction of the phylogenetic tree.

Genus	Virus (Abbreviation)	Accession number
bevemovirus	bellflower veinal mottle virus (BVMoV)	ARM19740.1
Brambyvirus	blackberry virus Y (BlVY)	YP_851006.1
Bymovirus	barley mild mosaic virus (BaMMV)	NP_604491.1/NP_604490.1
	barley yellow mosaic virus (BaYMV)	NP_148999.1/NP_149000.1
	oat mosaic virus (OMV)	NP_659025.1/NP_659026.1
	rice necrosis mosaic virus (RNMV)	YP_009175090.1/YP_009175089.1
	wheat yellow mosaic virus (WYMV)	NP_059449.1/NP_059448.1
Ipomovirus	cassava brown streak virus (CBSV)	ADR73022.1
	cucumber vein yellowing virus (CVYV)	YP_224077.1
	squash vein yellowing virus (SqVYV)	YP_001788991.1

Tab. 8.2: Virus sequences used for the construction of the phylogenetic tree.

Genus	Virus (Abbreviation)	Accession number
	sweet potato mild mottle virus (SPMMV)	NP_620656.1
	tomato mild mottle virus (TMMoV)	CCD57807.1
	Ugandan cassava brown streak virus (UCBSV)	ACN50007.1
Macluravirus	artichoke latent virus (ArLV)	YP_009129267.1
	broad-leafed dock virus A (BDVA)	AMN08808.1
	Chinese yam necrotic mosaic virus (CYNMV)	YP_006590058.1
Poacevirus	Caladenia virus A (CalVA)	YP_006666511.1
	sugarcane streak mosaic virus (SCSMV)	YP_003580191.1
	Triticum mosaic virus (TriMV)	YP_002956073.1
Potyvirus	Algerian watermelon mosaic virus (AWMV)	YP_001931956.1
	Apium virus Y (ApVY)	YP_004123951.1
	Arracacha mottle virus (AMoV)	YP_006522440.1
	Asparagus virus 1 (AV1)	YP_009110712.1
	banana bract mosaic virus (BBrMV)	YP_001427389.1
	Basella rugose mosaic virus (BaRMV)	YP_001427385.1
	bean common mosaic necrosis virus (BCMNV)	AAP38183.1
	bean common mosaic virus (BCMV)	AII73714.1
	bean yellow mosaic virus (BYMV)	NP_612218.1
	beet mosaic virus (BtMV)	NP_954611.1
	Bidens mosaic virus (BiMV)	YP_008877631.1
	Bidens mottle virus (BiMoV)	ABY86427.1
	blue squill virus A (BSVA)	YP_006990202.1
	Brugmansia mosaic virus (BruMV)	YP_007354880.1
	Brugmansia suaveolens mottle virus (BsMoV)	YP_003900466.1
	calla lily latent virus (CLLV)	YP_007969411.1
	Canna yellow streak virus (CaYSV)	YP_003208047.1
	carrot thin leaf virus (CTLV)	YP_009091824.1
	celery mosaic virus (CeMV)	YP_004376199.1
	chilli ringspot virus (CRSV)	YP_004875339.1
	chilli veinal mottle virus (ChiVMV)	NP_982308.1
	clover yellow vein virus (ClYVV)	BAM48928.1
	cocksfoot streak virus (CSV)	NP_620483.1
	Colombian Datura virus (CDV)	YP_007346986.1
	cowpea aphid-borne mosaic virus (CABMV)	NP_659018.1
	Cyrtanthus elatus virus A (CEVA)	YP_006383504.1
	daphne mosaic virus (DapMV)	YP_610949.1
	dasheen/vanilla mosaic virus (VanMV)	NP_613274.1
	East Asian Passiflora virus (EAPV)	BAM28738.1
	Freesia mosaic virus (FreMV)	YP_003587807.1

Tab. 8.2: Virus sequences used for the construction of the phylogenetic tree.

Genus	Virus (Abbreviation)	Accession number
	fritillary virus Y (FVY)	YP_001974419.1
	Gloriosa stripe mosaic virus (GSMV)	ABR88099.1
	Habenaria mosaic virus (HaMV)	ALE99280.1
	Hardenbergia mosaic virus (HarMV)	AHY61040.1
	Hippeastrum mosaic virus (HiMV)	YP_006382256.1
	Iranian johnsongrass mosaic virus (IJMV)	YP_006906026.1
	iris severe mosaic virus (ISMV)	YP_009224125.1
	Japanese yam mosaic virus (JYMV)	NP_051161.1
	johnsongrass mosaic virus (JGMV)	NP_619668.1
	keunjorong mosaic virus (KjMV)	YP_004934107.1
	konjac mosaic virus (KoMV)	YP_529485.1
	leek yellow stripe virus (LYSV)	BAE72832.1
	lettuce mosaic virus (LMV)	AIA66377.1
	lily mottle virus (LMoV)	BAJ10467.1
	lupinus mosaic virus (LuMV)	YP_004123732.1
	maize dwarf mosaic virus (MDMV)	NP_569138.1
	Moroccan watermelon mosaic virus (MWMV)	YP_001552410.1
	Narcissus degeneration virus (NDV)	YP_001019187.1
	Narcissus late season yellows virus (NLSYV)	AFQ95552.1
	Narcissus yellow stripe virus (NYLV)	YP_002308453.1
	onion yellow dwarf virus (OYDV)	NP_871002.1
	Ornithogalum mosaic virus (OrMV)	YP_006989380.1
	Panax virus Y (PanVY)	YP_003725718.1
	papaya leaf distortion mosaic virus (PLDMV)	NP_870995.1
	papaya ringspot virus (PRSV)	AAB23789.1
	passion fruit woodiness virus (PWV)	YP_004063671.1
	pea seed-borne mosaic virus (PSbMV)	NP_056765.1
	peanut mottle virus (PeMoV)	AHL84183.1
	Pennisetum mosaic virus (PenMV)	AGF29582.1
	pepper mottle virus (PepMoV)	NP_041276.1
	pepper severe mosaic virus (PepSMV)	YP_778468.1
	pepper veinal mottle virus (PVMV)	YP_002519375.1
	pepper yellow mosaic virus (PepYMV)	YP_003778216.1
	Peru tomato mosaic virus (PTV)	NP_787937.1
	plum pox virus (PPV)	NP_040807.1
	pokeweed mosaic virus (PkMV)	YP_008719787.1
	potato virus A (PVA)	NP_659729.1
	potato virus V (PVV)	NP_659008.1
	potato virus Y (PVY)	NP_056759.1

Tab. 8.2: Virus sequences used for the construction of the phylogenetic tree.

Genus	Virus (Abbreviation)	Accession number
	scallion mosaic virus (ScaMV)	NP_570725.1
	shallot yellow stripe virus (SYSV)	YP_331423.1
	sorghum mosaic virus (SrMV)	NP_659391.1
	soybean mosaic virus (SMV)	NP_072165.1
	sugarcane mosaic virus (SCMV)	NP_570724.1
	sunflower chlorotic mottle virus (SCMoV)	YP_003580192.1
	sweet potato feathery mottle virus (SPFMV)	NP_045216.1
	sweet potato latent virus (SPLV)	YP_007697620.1
	sweet potato virus 2 (SPV2)	YP_006382460.1
	sweet potato virus C (SPVC)	YP_004046670.1
	sweet potato virus G (SPVG)	YP_006493333.1
	Telosma mosaic virus (TelMV)	YP_001427386.1
	Thunberg fritillary mosaic virus (TFMV)	YP_254713.1
	tobacco etch virus (TEV)	NP_062908.1
	tobacco vein banding mosaic virus (TVBMV)	YP_001552409.1
	tobacco vein mottling virus (TVMV)	NP_056867.1
	tomato necrotic stunt virus (TNSV)	YP_006272948.1
	turnip mosaic virus (TuMV)	NP_062866.2
	Verbena virus Y (VVY)	YP_001931955.1
	watermelon mosaic virus (WMV)	YP_077181.1
	wild potato mosaic virus (WPMV)	NP_741959.1
	wild tomato mosaic virus (WTMV)	YP_001427388.1
	Wisteria vein mosaic virus (WVMV)	YP_271857.1
	yam mild mosaic virus (YMMV)	YP_006990077.1
	yam mosaic virus (YMV)	YP_022751.1
	yambean mosaic virus (YBMV)	YP_004936165.1
	Zantedeschia mild mosaic virus (ZaMMV)	YP_002308580.1
	zucchini tigre mosaic virus (ZTMV)	YP_008992091.1
	zucchini yellow mosaic virus (ZYMV)	NP_477522.1
roymovirus	rose yellow mosaic virus (RYMV))	YP_025106.1
Rymovirus	Agropyron mosaic virus (AgMV)	YP_006905847.1
	Hordeum mosaic virus (HoMV)	AAS65455.2
	ryegrass mosaic virus (RGMV)	NP_044727.1
Tritimovirus	brome streak mosaic virus (BrSMV)	NP_612585.1
	oat necrotic mottle virus (ONMV)	NP_932608.1
	tall oat-grass mosaic virus (TOgMV)	YP_008766766.1
	wheat eqlid mosaic virus (WEqMV)	YP_001468087.1
	wheat streak mosaic virus (WSMV)	NP_046741.1
	yellow oat-grass mosaic virus (YoGMV)	YP_009047077.1

In table 8.3, the accession numbers of sequences of the ORF and CP are listed, which were used for the calculation of the percentage identities of CeLV.

Tab. 8.3: Virus sequences used for the calculation of the ORF and CP percentage identity values.

		Accession number			
Genus	**Virus (Abbreviation)**	**ORF nt**	**ORF aa**	**CP nt**	**CP aa**
bevemovirus	bellflower veinal mottle virus (BVMoV)	KY491536.1	ARM19740.1	KY491536.1	ARM19740.1
Brambyvirus	blackberry virus Y (BlVY)	NC_008558.1	YP_851006.1	NC_008558.1	YP_851208.1
Bymovirus	barley yellow mosaic virus (BaMMV)	NC_002990.1/ NC_002991.1	NP_148999.1/ NP_149000.1	NC_002990.1	NP_734308.1
Ipomovirus	sweet potato mild mottle virus (SPMMV)	NC_003797.1	NP_620656.1	NC_003797.1	NP_734288.1
Macluravirus	Chinese yam necrotic mosaic virus (CYNMV)	NC_018455.1	YP_006590058.1	NC_018455.1	YP_006590102.1
Poacevirus	Triticum mosaic virus (TriMV)	NC_012799.1	YP_002956073.1	NC_012799.1	YP_002956096.1
Potyvirus	potato virus Y (PVY)	EU563512.1	ACD84569.1	EU563512.1	ACD84569.1
roymovirus	rose yellow mosaic virus (RYMoV)	NC_019031.1	YP_006905847.1	NC_019031.1	YP_006908988.1
Rymovirus	ryegrass mosaic virus (RGMV)	NC_001814.1	NP_044727.1	NC_001814.1	NP_734328.1
Tritimovirus	wheat streak mosaic virus (WSMV)	NC_001886.1	NP_046741.1	NC_001886.1	NP_734274.1

In table 8.4, the accession numbers of sequences of the ORF and CP are listed, which were used for the calculation of the percentage identities of CeLV.

Tab. 8.4: Virus sequences used for the calculation of the NIb percentage identity values.

		Accession number	
Genus	**Virus (Abbreviation)**	**NIb nt**	**NIb aa**
bevemovirus	bellflower veinal mottle virus (BVMoV)	KY491536.1	ARM19740.1
Brambyvirus	blackberry virus Y (BlVY)	NC_008558.1	YP_851207.1
Bymovirus	barley yellow mosaic virus (BaMMV)	NC_002990.1	NP_734307.1
Ipomovirus	sweet potato mild mottle virus (SPMMV)	NC_003797.1	NP_620656.1
Macluravirus	Chinese yam necrotic mosaic virus (CYNMV)	NC_018455.1	YP_006590101.1
Poacevirus	Triticum mosaic virus (TriMV)	NC_012799.1	YP_002956095.1
Potyvirus	potato virus Y (PVY)	EU563512.1	ACD84569.1
roymovirus	rose yellow mosaic virus (RYMV)	NC_019031.1	YP_006908987.1
Rymovirus	ryegrass mosaic virus (RGMV)	NC_001814.1	NP_734327.1
Tritimovirus	wheat streak mosaic virus (WSMV)	NC_001886.1	NP_734273.1

8.3 Vector- and plasmid maps

8.3.1 Vector pDIVA KX665539

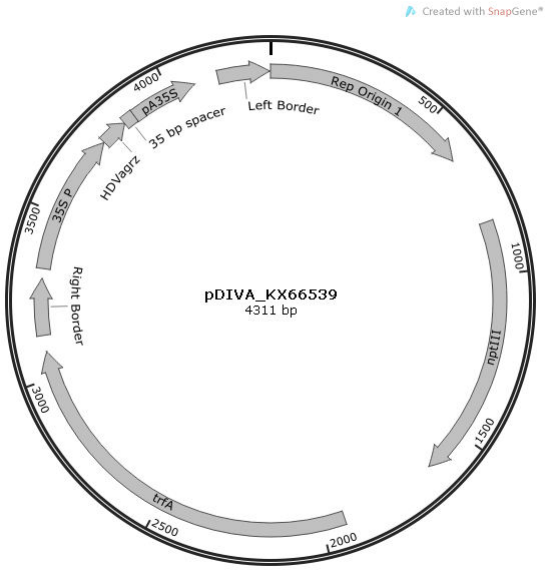

Fig. 8.1: Vector map of pDIVA. Rep Origin 1: Origin of Replication RK2 OriV, nts 1-630; **nptIII:** kanamycin resistance, nts 839-1633; **trfA:** Plasmid replication initiator protein, nts 1932-3080; **Right border:** 3130-3299; **35S P:** CaMV 35S promoter, nts 3330-3754; **HDVagrz:** *Hepatitis delta virus* self-cleaving antigenomic "core" ribozyme, nts 13.755-13.839; **35 bp spacer:** 13.840-13.874; **pA35S:** polyadenylation signal of CaMV, nts 13.875-14.079; **Left border:** nts 14.153-14.309.

8.3.2 Full-length cDNA clones of CeLV and CeMV

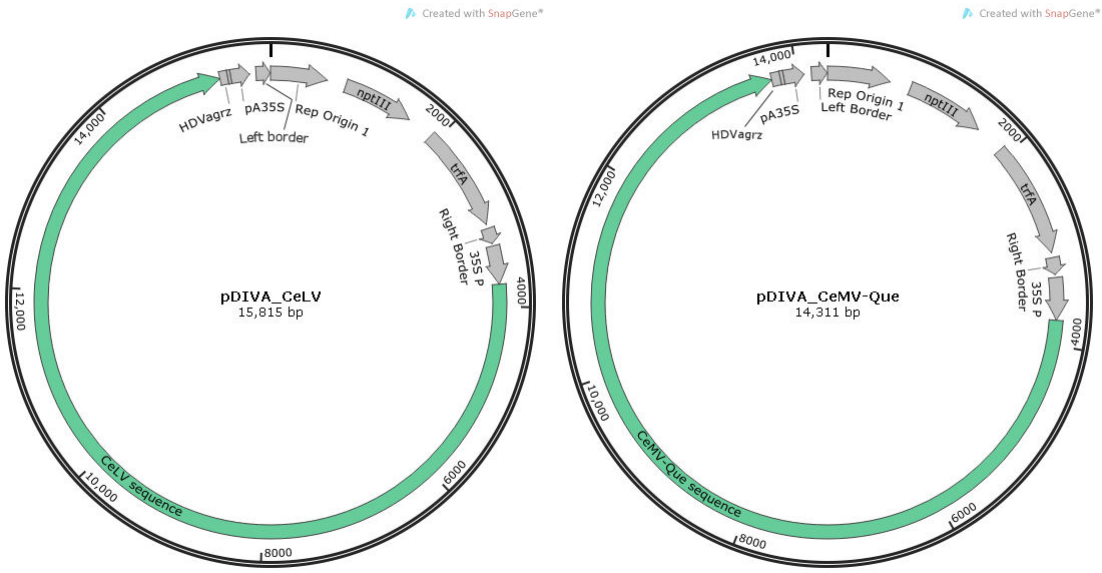

Fig. 8.2: Vector maps of CeLV (left) and CeMV (right) full-length cDNA clones. Rep Origin 1: Origin of Replication RK2 OriV; **nptIII:** kanamycin resistance; **trfA:** Plasmid replication initiator protein; **35S P:** CaMV 35S promoter; **HDVagrz:** *Hepatitis delta virus* antigenomic self-cleaving ribozyme; **pA35S:** polyadenylation signal of CaMV. Not all features of pDIVA are listed in the maps.

8.4 Oligonucleotides used for cloning of the silencing constructs

In the following table 8.5 all oligonucleotides for the construction of the silencing clones are shown.

Tab. 8.5: Oligonucleotides sense (s) and antisense (as) used for the amplification of the silencing constructs. Underlined parts are complementary to pDIVA or template sequence. Complementary stretches within overlapping primers are italicized and bold. Final constructs are bold. White or grey marked rows belong to cloning of the same construct.

Oligonucleotide name	Oligonucleotide sequence	Template	Use of oligonucleotide & construct
pDIVA_as	CCTCTCCAAATGAAATGAACTTCCTTATATAG	pDIVA	Amplification of **pDIVA** for Gibson Assembly
pDIVA_s	GGGTCGGCATGGCATCTCCACCTCCTC		
pDIVA_s	GGGTCGGCATGGCATCTCCACCTCCTC	CeLV full-length clone	Construction of CeLV GDD (Δ stop)
CeLVSilenceGDDas	GGGTCGGCATGGCATCTCCACCTCCTC		
pDIVA_s	***GGGTCGGCATG***GCATCTCCACCTCCTC	CeLV GDD Δ stop	Introduction stop into **CeLV GDD**
Stop_CeLV_GDD (as)	***CATGCCGACCCT***TAATCATCTCCATTCAC		
pDIVA_s	GGGTCGGCATGGCATCTCCACCTCCTC	CeLV full-length clone	Construction of CeLV DECH (Δ stop)
CeLVSilenceDXas	ATGACATTCATCAGCTAAAATATAATCAA		
pDIVA_s	***GGGTCGGCATG***GCATCTCCACCTCCTC	CeLV DECH Δ stop	Introduction stop into **CeLV DECH**
Stop_CeLV_DECH (as)	***CATGCCGACCCT***TAATGACATTCATCAGC		
s_CeLV_DECH-GDD	CATTTCATTTGGAGAGGGATGAATGTCATGTTGTAAC	*CeLV full-length clone*	Construction of CeLV DECH-GDD (Δ leader Δ stop) for Gibson Assembly into empty pDIVA
as_CeLV_DECH-GDD	GAGATGCCATGCCGACCCATCATCTCCATTCACAC		
L_DE-GDos	***AAGCTTATCGATTAGGAG***ATATAACAATGGATGAATGTCATGTTG	CeLV DECH-GDD Δ leader Δ stop	Introduction of leader sequence into CeLV DECH-GDD (Δ leader Δ stop)
L_PAPA_asoverl (as)	***CTCCTAATCGATAAGCTT***GGATCCCCTCTCCAAATGAAATG		
pDIVA_s	***GGGTCGGCATG***GCATCTCCACCTCCTC	CeLV DECH-GDD Δ stop	Introduction of stop into **CeLV DECH-GDD**
Stop_CeLV_GDD	***CATGCCGACCCTT***AATCATCTCCATTCAC		

Tab. 8.5: Oligonucleotides sense (s) and antisense (as) used for the amplification of the silencing constructs. Underlined parts are complementary to pDIVA or template sequence. Complementary stretches within overlapping primers are italicized and bold. Final constructs are bold. White or grey marked rows belong to cloning of the same construct.

Oligonucleotide name	Oligonucleotide sequence	Template	Use of oligonucleotide & construct
CeLV_DPPH_s	***CGATTAGGAG***ATATAACAATGGACCCACCCCATGTG	CeLV-DECH	Construction of **CeLV DPPH-DECH**
L_PAPA_asoverl (as)	***CTCCTAATCG***ATAAGCTTGGATCCCCTCTCCAAATGAAATG		
CDECHDP1s	***TTATCGATTAGGAGA***TATAACAATGGTGAGGAATTGTGAAAC	CeLV DECH	Construction of **CeLV DECH Δ P1**
CDECHDP1as	***TCTCCTAATCGATAA***GCTTGGATCCCCTCTCCAAATGAAATG		
CeLV_SIAT_s	***CGATTAGGAG***ATATAACAATGTCAATAGCTACCATGG	CeLV-DECH	Construction of **CeLV SIAT-DECH**
L_PAPA_asoverl (as)	***CTCCTAATCG***ATAAGCTTGGATCCCCTCTCCAAATGAAATG		
TAA_Mama_s	TAAGGGTCGGCATGGCATCTCC	CeLV-DECH	Construction of **CeLV KIDS**
CeLV_KIDS_as	CGAATCGATTTTATGTTTTTCTGC		
CeLV_DPPH_s	***CGATTAGGAG***ATATAACAATGGACCCACCCCATGTG	CeLV-AIVG	Construction of **CeLV DPPH-AIVG**
L_PAPA_asoverl (as)	***CTCCTAATCG***ATAAGCTTGGATCCCCTCTCCAAATGAAATG		
CeLV_SIAT_s	***CGATTAGGAG***ATATAACAATGTCAATAGCTACCATGG	CeLV-AIVG	Construction of **CeLV SIAT-AIVG**
L_PAPA_asoverl (as)	***CTCCTAATCG***ATAAGCTTGGATCCCCTCTCCAAATGAAATG		
pDIVA_s	GGGTCGGCATGGCATCTCCACCTCCTC	*CeLV full-length clone*	Construction of CeLV AIVG (Δ stop)
CeLV_AIVG.G_as	ACCAACAATTGCTCTCTTAAAATGTTC		
pDIVA_s	***GGGTCGGCATG***GCATCTCCACCTCCTC	CeLV AIVG Δ stop	Introduction stop into **CeLV AIVG**
Stop_CeLV_IVGG (as)	***CATGCCGACCC***TTAACCAACAATTGCTC		
TAA_Mama_s	TAAGGGTCGGCATGGCATCTCC	CeLV-DECH	Construction of **CeLV TLLT**
CeLV_TLLT_as	TGTTAACAGAGTCTGCAATTGCCTAA		
TAA_Mama_s	TAAGGGTCGGCATGGCATCTCC	CeLV-DECH	Construction of **CeLV HVGY**
CeLV_HVGY_as	ATAACCAACATGAATGTGATATCTAGAACC		

Tab. 8.5: Oligonucleotides sense (s) and antisense (as) used for the amplification of the silencing constructs. Underlined parts are complementary to pDIVA or template sequence. Complementary stretches within overlapping primers are italicized and bold. Final constructs are bold. White or grey marked rows belong to cloning of the same construct.

Oligonucleotide name	Oligonucleotide sequence	Template	Use of oligonucleotide & construct
CeLV_SIAT_s	***CGATTAGGAG***ATATAACAATGTCAATAGCTACCATGG	CeLV-HVGY	Construction of **CeLV SIAT-HVGY**
L_PAPA_asoverl (as)	***CTCCTAATCG***ATAAGCTTGGATCCCCTCTCCAAATGAAATG		
TAA_Mama_s	TAAGGGTCGGCATGGCATCTCC	CeLV-HVGY	Construction of **CeLV EGPS**
CeLV_EGPS_as	CGAGGGTCCCTCCTTTGTTTTAG		
TAA_Mama_s	TAAGGGTCGGCATGGCATCTCC	CeLV-HVGY	Construction of **CeLV KARA**
CeLV_KARA_as	TGCTCGTGCCTTCCGTTGTTG		
TAA_Mama_s	TAAGGGTCGGCATGGCATCTCC	CeLV-DECH	Construction of **CeLV VNPG**
CeLV_VNPG_as	CCCTGGGTTCACACAAATTTCTTG		
CDECHDP1s	***TTATCGATTAGGAGA***TATAACAATGGTGAGGAATTGTGAAAC	CeLV AIVG	Construction of **CeLV AIVG Δ P1**
CDECHDP1as	***TCTCCTAATCGATAA***GCTTGGATCCCCTCTCCAAATGAAATG		
pDIVA_s	***GGGTCGGCATGGCATC***TCCACCTCCTC	CeLV AIVG	Construction of **CeLV IEKY**
CeLVst_IEKYV_P1 (as)	***GATGCCATGCCGACCC***TTAATATTTTTCAATATG		
L_CeLV-CP_s_over	***AAGCTTATCGATTAGGAG***ATATAACAATGAGTCACTACCC	*CeLV full-length clone*	Construction of **CeLV CP** (3'-UTR included)
L_PAPA_asoverl (as)	***CTCCTAATCGATAAGCTT***GGATCCCCTCTCCAAATGAAATG		
pDIVA_s	GGGTCGGCATGGCATCTCCACCTCCTC	PPV full-length clone	Construction of PPV GDD (Δ stop)
PPVSilenceGDDas	ATCATCACCATTCACAAAGTACCG		
pDIVA_s	***GGGTCGGCAT***GGCATCTCCACCTCCTC	PPV GDD (Δ stop)	Construction of **PPV GDD**
Stop_PPV_GDD (as)	***CATGCCGACCC***CTAATCATCACCATTCAC		
s_PPV_DECH-GDD	CATTTCATTTGGAGAGGGATGAATGTCACGTGCATGATG	PPV full-length clone	PPV DECH-GDD (Δ leader Δ stop) for Gibson Assembly into pDIVA
as_PPV_DECH-GDD	GATGCCATGCCGACCCATCATCACCATTCACAAAGTAC		

Tab. 8.5: Oligonucleotides sense (s) and antisense (as) used for the amplification of the silencing constructs. Underlined parts are complementary to pDIVA or template sequence. Complementary stretches within overlapping primers are italicized and bold. Final constructs are bold. White or grey marked rows belong to cloning of the same construct.

Oligonucleotide name	Oligonucleotide sequence	Template	Use of oligonucleotide & construct
L_PPV_DE-GD_o_s	***AAGCTTATCGATTAGGAG***ATATAACAATGGATGAATGTCACGTGC	PPV DECH-GDD (Δ leader Δ stop)	Introduction of leader sequence into PPV DECH-GDD (Δ leader Δ stop)
L_PAPA_asoverl (as)	***CTCCTAATCGATAAGCTT***GGATCCCCTCTCCAAATGAAATG		
pDIVA_s	***GGGTCGGCATG***GCATCTCCACCTCCTC	PPV DECH-GDD (Δ stop)	Introduction of stop into **PPV DECH-GDD**
Stop_PPV_GDD (as)	***CATGCCGACCCC***TAATCATCACCATTCAC		
pDIVA_s	GGGTCGGCATGGCATCTCCACCTCCTC	PPV full-length clone	Construction of PPV DECH (Δ stop)
PPVSilenceDXas	GTGACATTCATCAAAAATGATGCAC		
pDIVA_s	***GGGTCGGCATG***GCATCTCCACCTCCTC	PPV DECH (Δ stop)	Introduction of stop into **PPV DECH**
Stop_PPV_DECH	***CATGCCGACCC***CTAGTGACATTCATCAA		
PPV_FANT_s	ATATAACAATGTTTGCAAACACAAGCG	PPV DECH	Construction of **PPV FANT-DECH**
L_PAPA_asoverl (as)	CTCCTAATCGATAAGCTTGGATCCCCTCTCCAAATGAAATG		
pDIVA_s	GGGTCGGCATGGCATCTCCACCTCCTC	PPV full-length clone	Construction of PPV YLVG (Δ stop)
PPV_LVG.G_as	TCCAACCAGGTATGTTTTCATATTTG		
pDIVA_s	***GGGTCGGCATG***GCATCTCCACCTCCTC	PPV YLVG (Δ stop)	Introduction of stop into **PPV YLVG**
Stop_PPV_LVGG (as)	***CATGGCATGCCGACCC****CTA*TCCAACCAGGTATG		
PPV_FANT_s	ATATAACAATGTTTGCAAACACAAGCG	PPV YLVG	Construction of **PPV FANT-YLVG**
L_PAPA_asoverl (as)	CTCCTAATCGATAAGCTTGGATCCCCTCTCCAAATGAAATG		
PPV_KVSK_s	***CGATTAGGAG***ATATAACAATGAAGGTTTCTAAAAAGC	PPV YLVG	Construction of **PPV KVSK-YLVG**
L_PAPA_asoverl (as)	***CTCCTAATCG***ATAAGCTTGGATCCCCTCTCCAAATGAAATG		
s_PPV_HC-Pro_PAP	CATTTCATTTGGAGAGGACACGCTGAAATCACCAGTC	pBI HC-Pro	PPV HC-Pro (Δ leader) for Gibson Assembly into pDIVA
as_PPV_HC-Pro_Ma	GATGCCATGCCGACCCCTATCCAACCAGGTATG		

Tab. 8.5: Oligonucleotides sense (s) and antisense (as) used for the amplification of the silencing constructs. Underlined parts are complementary to pDIVA or template sequence. Complementary stretches within overlapping primers are italicized and bold. Final constructs are bold. White or grey marked rows belong to cloning of the same construct.

Oligonucleotide name	Oligonucleotide sequence	Template	Use of oligonucleotide & construct
L_HC-Pro_s*over (s)	***AAGCTTATCGATTAGGAG***ATATAACAATGGACCCAGGCAAAC	pCB HC-Pro (Δ leader)	Introduction of leader sequence into **PPV HC-Pro**
L_PAPA_asoverl (as)	***CTCCTAATCGATAAGCTT***GGATCCCCTCTCCAAATGAAATG		
MAMA (s)	***GGGTCGGCATGGCATC***TCCACCTCCTC	PPV YLVG	Construction of **PPV P1**
Stop_PPV_P1 (as)	***GATGCCATGCCGACCC***CTAGTAGTGGATTATCTC		
L_PPV-CP_s_over	***AAGCTTATCGATTAGGAG***ATATAACAATGGCTGACGAAAG	PPV full-length clone	Construction of **PPV CP**
L_PAPA_asoverl (as)	***CTCCTAATCGATAAGCTT***GGATCCCCTCTCCAAATGAAATG		

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10 Curriculum Vitae

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08.2004-06.2007	Max-Planck-Gymnasium, Delmenhorst Abschluss: Allgemeine Hochschulreife
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11 Publication list

Publications without peer review process

1. **Rose, H., Döring, I., Vetten, H.J., Menzel, W., Richert-Pöggeler, K. R., Maiß, E. (2017).** Celery latent virus: Difficulties and pitfalls in sequence determination and construction of an infectious full-length clone. In: Proceedings of the 6th Joint Meeting of the DPG-working group "Virus Diseases of Plants" and the “Nederlandse Kring voor Plantevirologie”, 27.-28.03.2017, Rheinisch Friedrich-Wilhelms Universität Bonn, (talk presentation)

2. **Rose, H., Maiß, E. (2017).** Complete sequence determination and construction of an infectious full-length clone of a celery mosaic virus isolate from Quedlinburg (Germany). In: Proceedings of the 6th Joint Meeting of the DPG-working group "Virus Diseases of Plants" and the “Nederlandse Kring voor Plantevirologie”, 27.-28.03.2017, Rheinische Friedrich-Wilhelms Universität Bonn, (poster presentation)

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4. **Schiwek, S., Rose, H., Maiß, E. (2017).** Studies on the identification of a silencing suppressor of celery latent virus. In: Proceedings of the 6th Joint Meeting of the DPG-working group "Virus Diseases of Plants" and the “Nederlandse Kring voor Plantevirologie”, 27.-28.03.2017, Rheinische Friedrich-Wilhelms Universität Bonn, (poster presentation)

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8. **Rose, H., Büttner, C., von Bargen, S., Langer, J., Winter, S., Maiß, E. (2015).** Erste Untersuchungen zum Nachweis von Potyviren aus afrikanischen Nachtschattengewächsen (*Solanum* spp.). In: Proceedings of the 47th Annual Meeting of the DPG-working group "Virus Diseases of Plants", 16.-17.03.2015, Humboldt-Universität zu Berlin, (poster presentation)

9. **Rose, H., Eikenberg, I., Menzel, W., Vetten, H.J., Maiß, E. (2014).** Celery latent virus: Ein potentielles Mitglied der *Potyviridae* mit einem N-terminal lokalisierten Signalpeptid. 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, (talk presentation)

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12. **Rose, H., Heinze, C., Maiss, E. (2012).** Labelling of Tobacco mosaic virus with gfp (green fluorescent protein). In: Proceedings of the 58th German Plant Protection Conference, 10.-14.09.2012, Technische Universität Braunschweig, (talk presentation)

13. **Lesker, T., Göing, J., Rose, H., Schneider, C., Korte, J., Maiß, E. (2010).** DsRNA screening – Isolation, molecular characterization and phylogenetic analysis of possible dsRNA viruses in vegetables, herbs and ornamentals. In: Proceedings of the 57th German Plant Protection Conference, 06.-09.09.2010, Humboldt-Universität zu Berlin, (poster presentation)

Bachelor and Master thesis

Rose, H. (2012). Labelling of Tobacco Mosaic Virus (TMV, OhioV) Using Reporter Genes with Regard to Movement Studies in *Nicotiana benthamiana*. Master thesis of the Institute of Plant Diseases and Plant Protection, Leibniz Universität Hannover

Rose, H. (2010). First molecular analysis of a cryptic virus from Japanese green. Bachelor thesis of the Institute of Plant Diseases and Plant Protection, Leibniz Universität Hannover