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Construction of infectious full-length cDNA clones of apple viruses and plant viral vector development

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Is viral infection passive or active?

Abstract

Apple chlorotic leaf spot virus (ACLSV) and *Apple stem pitting virus* (ASPV) are important viral pathogens in apple. The aim of the present work was to construct full-length cDNA clones of ACLSV and ASPV and to agroinoculate apple seedlings with the constructed infectious clones using a newly developed vacuum infiltration method. A further goal was to explore and create viral vectors based on the obtained infectious full-length cDNA clones of ACLSV.

In the thesis, the full-length cDNA clones of ACLSV and ASPV were constructed using different methods. The methods contained circular polymerase extension cloning (CPEC), Gibson assembly and In-Fusion cloning. In total 17 full-length cDNA clones of ACLSV and ASPV were obtained. Four of the 17 clones were infectious on *Nicotiana occidentalis* 37B, i.e. pIF3-15, pIF3-19, pIF14-23 and pIF4-4. The viral genomic cDNAs in these infectious clones were completely sequenced, and the sequence data were analyzed by alignment with published sequences in NCBI. The results indicated that three isolates of ACLSV were rescued: ACLSV isolate 38/85A (pIF3-15), 38/85B (pIF3-19) and (36)/88 (pIF14-23). One ASPV isolate was rescued: ASPV 40/87.

A protocol of agroinoculation of apple seedlings by vacuum infiltration was developed to inoculate the infectious clones (pIF3-15, pIF3-19, pIF14-23 and pIF4-4) to apple seedlings. In the protocol, the treatment of seedlings, preparation of inocula and parameters of vacuum infiltration were evaluated. The highest PCR-positive rate (infection) of 78% (11/14), 100% (11/11), 25% (2/8) and 50% (9/18) were observed for the infectious clones of pIF3-15, pIF3-19, pIF14-23 and pIF4-4, respectively. The infection of virus was determined by RT-PCR. The existence of viral particles in PCR-positive plants was determined by immunosorbent electron microscopy.

To explore and develop plant viral vectors based on ACLSV, marker genes of Emerald GFP, mCherry or iLov were inserted into the genomic cDNA of ACLSV. Nine plasmids with

marker genes were constructed using three different strategies, including pIF13-9, pIF18-2, pIF25-7, pG11-15, pIF24-6, pIF23-1, pIF16-1, pIF20-16 and pIF27-10. After agroinoculation of *N. occidentalis* 37B with the constructed plasmids, it was found that deletion of marker genes in pIF13-9 and pG11-15 occurred due to homologous recombination between duplicated fragments of ACLSV genomic cDNA. Typical ACLSV symptoms were observed on the test-plants inoculated with pIF13-9 and pG11-15. By western blot, viral proteins of CP of identical size with wild type virus (pIF3-19) were detected for the two constructs in symptomatic plants. In one trial in winter, the pIF25-7 caused systemic infection in one plant. The other plasmids of pIF18-2, pIF24-6, pIF23-1, pIF16-1, pIF20-16 and pIF27-10 did not cause local or systemic infection in any test-plant.

Zusammenfassung

Apple chlorotic leaf spot virus (ACLSV) und *Apple stem pitting virus* (ASPV) sind wichtige virale Erreger in Apfelkulturen. Das Ziel dieser Arbeit war die Konstruktion von Volllängen cDNA Klonen von ACLSV und ASPV und deren Infektion an Apfelsämlingen durch eine neu entwickelte Vakuuminfiltrations-Methode zur Agroinokulation. Ein weiteres Ziel war die Untersuchung und Konstruktion viraler Vektoren basierend auf den hergestellten infektiösen Volllängen cDNA Klonen von ACLSV.

In dieser Arbeit wurden Volllängen cDNA Klone von ACLSV und ASPV über verschiedene Methoden hergestellt. Diese Methoden waren das *Circular Polymerase Extension Cloning* (CPEC), Gibson Assemblierung und In-Fusion Klonierung. Insgesamt konnten 17 Volllängen cDNA Klone von ACLSV und ASPV hergestellt werden. Vier dieser 17 Klone waren infektiös auf *Nictoiana occidentalis* 37B (pIF3-15, pIF3-19, pIF14-23 und pIF4-4). Die virale genomische cDNA dieser Klone wurde vollständig sequenziert und mit bereits veröffentlichen Sequenzen in NCBI verglichen. Die Ergebnisse deuteten darauf hin, dass drei verschiedene Isolate von ACLSV vorlagen: ACLSV Isolate 38/85A (pIF3-15), 38/85B (pIF3-19) und (36)/88 (pIF14-23). Von ASPV konnte ein Isolat identifiziert werden (ASPV 40/87).

Um Apfelsämlinge mit den infektiösen Klonen pIF3-15, pIF3-19, pIF14-23 und pIF4-4 inokulieren zu können, wurde ein Vakuuminfiltrations-Protokoll zur Agroinokulation entwickelt. Für das Protokoll wurden die Behandlung der Sämlinge, die Präparation des Inokulums und die Parameter der Vakuuminfiltration optimiert. Für die infektiösen Klone pIF3-15, pIF3-19, pIF14-23 bzw. pIF4-4 wurden 78% (11/14), 100% (11/11), 25% (2/8) und 50% (9/18) positiv getesteter Sämlinge beobachtet. Die Virusinfektion wurde dabei mit Hilfe von RT-PCR überprüft. Das Vorkommen viraler Partikel in PCR-positiven Pflanzen wurde über Immun-Elektronenmikroskopie bestätigt.

Um pflanzliche virale Vektoren, basierend auf ACLSV zu entwickeln und zu untersuchen, wurden Markergene von Emerald GFP, mCherry oder iLov in die genomische cDNA von ACLSV inseriert. Insgesamt neun Plasmide mit Markergenen wurden über drei verschiedene Klonierungsstrategien hergestellt, die mit pIF13-9, pIF18-2, pIF25-7, pG11-15, pIF24-6, pIF23-1, pIF16-1, pIF20-16 und pIF27-10 bezeichnet wurden. Nach der Agroinokulation von *N. occidentalis* 37B mit den Plasmidkonstrukten konnte festgestellt werden, dass in pIF13-9 und pG11-15 eine Deletion der Markergene über homologe Rekombination zwischen duplizierten Fragmenten der genomischen cDNA von ACLSV stattgefunden hat. Auf den mit pIF13-9 und pG11-15 inokulierten Testpflanzen konnten charakteristische ACLSV Symptome beobachtet werden. Über Western Blot konnten in den symptomatischen Pflanzen aus beiden Konstrukten virale CP Proteine mit der identischen Größe des Wildtyp Virus‘ (pIF3-19) detektiert werden. In einem Versuchsdurchlauf im Winter verursachte pIF25-7 eine systemische Infektion in einer einzigen Pflanze. Die anderen Plasmide von pIF18-2, pIF24-6, pIF23-1, pIF16-1, pIF20-16 und pIF27-10 verursachten weder lokale noch systemische Infektionen in den Testpflanzen.

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Abbreviations

Items	Abbreviations	Full text
Viruses	ACLSV	*Apple chlorotic leaf spot virus*
	ALSV	*Apple latent spherical virus*
	ALSV	*Apple latent spherical virus*
	AMV	*Alfalfa mosaic virus*
	ApMV	*Apple mosaic virus*
	ASGV	*Apple stem grooving virus*
	ASPV	*Apple stem pitting virus*
	BYDV	*Barley yellow dwarf virus*
	CaMV	*Cauliflower mosaic virus*
	CLBV	*Citrus leaf blotch virus*
	CMV	*Cucumber mosaic virus*
	CPMV	*Cowpea mosaic virus*
	CTV	*Citrus tristeza virus*
	GRSPaV	*Grapevine rupestris stem pitting-associated virus*
	GVA	*Grapevine virus A*
	GVB	*Grapevine virus B*
	MRFV	*Maize rayado fino virus*
	PLDMV	*Papaya leaf distortion mosaic virus*
	PPV	*Plum pox virus*
	PVX	*Potato virus X*
	PVY	*Potato virus Y*
	TMV	*Tobacco mosaic virus*
	ToBMV	*Tomato blistering mosaic virus*
Units	bp	Base pair
	cm	Centermeter
	ca.	Circa
	hPa	Hectopascal
	kb	Killobase
	kDa	Killodalton
	min	Minute
	ml	Mililiter
	mm	Milimeter
	mM	Millimolar
	ng	Nanogram
	nm	Nanometer
	nt	Nucleotide

Items	Abbreviations	Full text
	nts	Nucleotides
	rpm	Revolutions per minute
	sec	Second
	V	Volt
	µF	Microfarad
	µg	Microgram
	µl	Microliter
	µM	Micromolar
Others	APS	Ammonium persulfate
	CP	Coat protein
	CPEC	Circular polymerase extension cloning
	DNA	Deoxyribonucleic acid
	DTT	Dithiothreitol
	EDTA	Ethylenediaminetetraacetic acid
	EmGFP	Emerald-GFP
	GFP	Green fluorescent protein
	HDVpA	Hepatitis delta viral ribozyme followed by a CaMV 35S polyadenylation signal
	ISEM	Immunosorbent electron microscopy
	JKI	Julius Kuehn Institute
	LB	Luria-Bertani medium
	MP	Movement protein
	ORF	Open reading frame
	PAG	Polyacrylamide gel
	PAGE	Polyacrylamide gel electrophoresis
	PCR	Polymerase chain reaction
	polyA	Polyadenylated
	PRT	Premium reverse transcription
	PRTase	Premium reverse transcriptase
	RdRp	RNA-dependent RNA ploymerase
	RNA	Ribonucleic acid
	RT	Reverse transcription
	RTase	Reverse transcriptase
	SDS	Sodium dodecyl sulfate
	SOC	Super optimal broth with catabolite repression (SOC)
	spp.	Species
	ssDNA	Single-stranded DNA
	ssRNA	Single-stranded RNA
	subsp.	Subspecies
	TEMED	Tetramethylethylenediamine
	TGB	Triple gene block
	UTR	Untranslated region
	YEB	Yeast extract broth

1. Introduction

Apple chlorotic leaf spot virus (ACLSV) and *Apple stem pitting* virus (ASPV) were first described in the 1950s (Luckwill and Campbell 1959; Smith 1954). They are latent (eliciting no symptom) in most commercial apple cultivars (Eastwell and Howell 2014a, b; Jelkmann and Paunovic 2011; Myrta et al. 2011). Without use of certificaion systems (EPPO 1999) latent viruses are propagated and get widely distributed in orchards over time. Therefore, these viruses are found throughout pome fruit-growing regions in the world.

This work focuses on the construction of infectious full-length cDNA clones of ACLSV and ASPV. Furthermore experiments were conducted to develop a plant viral expression vector based on ACLSV clones.

1.1 Properties of ACLSV and ASPV

1.1.1 Virion properties

ACLSV is the type species of the genus *Trichovirus* within the family *Betaflexiviridae*. It has virions of very flexuous filaments, usually 640-890 nm in length and 10-12 nm in diameter, with distinct cross banding. ACLSV virions contain a single molecule of linear positive single-stranded (ss) RNA about 7500 nucleotides (nts) in size excluding the polyadenylated (polyA) tract at 3' terminus, accounting for about 5% of the particle weight (Adams et al. 2012). The genome has three slightly overlapping open reading frames (ORFs). ORF1, 2 and 3 encode a RNA-dependent RNA polymerase (RdRp, ca. 217 kDa), a movement protein (MP, ca. 50 kDa), and a coat protein (CP, ca. 22 kDa), respectively (German et al. 1990; Sato et al. 1993). ACLSV shows a very large molecular variability among isolates. The genomes show an overall identity between 76 and 82% in their nucleotide sequences. The CP is more conserved (87-93% identity) than the MP (77-85% identity) (Adams et al. 2012; Candresse et al. 1996; German-Retana et al. 1997).

ASPV is the type species of the genus *Foveavirus* within the family *Betaflexiviridae.* Its virions are flexuous filaments, 800-1000 nm in length and 12-15 nm in diameter with a surface pattern of cross-banding (Yanase et al. 1988). The virions contain a single molecule of positive-sense ssRNA of around 9500 nts excluding the polyA tail at 3' terminus (Jelkmann 1994; Jelkmann et al. 1991). The genome has five ORFs. The ORF1 encodes an RdRp (247 kDa). The ORFs 2-4 as triple gene block (TGB) encode proteins of 25, 13 and 8 kDa, respectively, constituting putative MP. The ORF5 encodes a CP (44 kDa).

1.1.2 Host range

The natural hosts of ACLSV mainly include *Rosaceae*. For example, ACLSV infects several *Prunus* species, including peach, apricot, almond, cherry, and plum (Myrta et al. 2003), blackthorn (*P. spinosa*) (Sweet 1980), Himalayan wild cherry (*P. cerasoides*) (Rana et al. 2008) and dwarf flowering almond (*P. glandulosa* 'Sinensis') (Spiegel et al. 2005). It also occurs in *Malus* species, such as cultivated apples and ornamental species of *M. platycarpa*, *M. floribunda*, *M. robusta*, *M. coronaria* (Desvignes et al. 1999). It is detected in hawthorn (*Crataegus spp.*) (Sweet 1980), pear (*Pyrus spp.*) and quince (*Cydonia oblonga*) too (Lister 1970; Németh 1986).

The experimental host range of ACLSV is limited to a few herbaceous species, including *Chenopodium quinoa*. *C. amaranticolor*, *Leguminosae* and *Nicotiana occidentalis* (Lister et al. 1965; Yoshikawa 2001).

The natural host range of ASPV is largely restricted to *Maloideae*, such as *Malus* spp., *Crataegus* spp., *Sorbus* spp., *Pyrus* spp. [*P. communis* (European pear), *P. serotinia* (Japanese pear), *P. ussuriensis* (Chinese pear), and *P. sinkiangensis*)], and *Cydonia oblonga* (quince) (Dhir et al. 2010; Ma et al. 2008; Mathioudakis et al. 2010; Mathioudakis et al. 2012). The most commonly used experimental hosts are *N. occidentalis* 37B and *N. occidentalis* subsp. obliqua (Koganezawa and Yanase 1990; Van der Meer 1985).

1.1.3 Symptoms and impacts

ACLSV causes cytopathological changes in infected cells to varying extents (Adams et al. 2012; Ohki et al. 1989). The virions are found in mesophyll, phloem and parenchyma cells of leaves and roots. They accumulate in the cytoplasm or between tonoplast and cytoplasm in bundles or paracrystalline aggregates. They were found in the nucleus as well. No inclusion bodies are formed.

Common isolates of ACLSV do not cause observable symptoms in most commercial cultivars of pome and stone fruit trees currently in production. In some susceptible species or cultivars, the symptom severity depends on the plant species and virus isolates, and symptoms appear mainly on leaves, fruits and more rarely on the trunk (Myrta et al. 2011; Németh 1986). For example, severe isolates elicit a apricot disease of 'pseudopox' (Desvignes and Boyé 1988), deforming the fruit. Some isolates induce 'false plum pox' on leaves and fruit of some plum cultivars (Jelkmann and Kunze 1994; Lebas et al. 2003). *Maruba kaido* (*Malus prunifolia* var. ringo) is susceptible to ACLSV, causing the particular occurrence of the apple topworking disease in Japan. The species of *M. sylvestris* cv. R12740-7A, *M. platycarpa*, *M. hupehensis*, *M. prunifolia* var. ringo can be used as diagnostic indexing varieties. In addition, ACLSV is associated with apple russet ring disease and pear ring pattern mosaic (Cropley et al. 1963; Desvignes and Boyé 1988; Yanase 1974).

ACLSV causes considerable yield losses in apple. In mixed infections with ASPV and *Apple stem grooving virus* (ASGV), a yield loss of 12 to 30% was observed on *Malus domesticus* cv. Golden Delicious in USA (Meijneke et al. 1973; Van Oosten et al. 1982). It has been reported that in China mixed infection with ASGV led to a yield loss up to 40% (Wu et al. 1997).

ASPV elicits a severe derangement of the cytology of infected cells. The virions of ASPV are found in mesophyll, epidermal and vascular parenchyma cells of infected plants. They accumulate in bundles in the cytoplasm, but no specific cytopathic structures or inclusion are formed (Adams et al. 2012; Koganezawa and Yanase 1990).

ASPV is latent in most common commercial apple cultivars, but susceptible cultivars do react with a variety of symptoms. For example, ASPV causes xylem pitting in the stem of *M. pumila* cv. Virginia crab, and elicits epinasty and decline on *M. pumila* cv. Spy 227. It also causes symptoms on susceptible pear and quince cultivars. For example, on infected *P. communis*, symptoms of narrow chlorotic bending of veins and red mottling are expressed (Cameron 1989). It induced symptoms of black sooty lines and rings bordering veins, pale yellow spots on leaves and fruit malformations on susceptible quince cultivars (Desvignes 1971; Paunovic 1994). These sensitive varieties are used as serological indicators (EPPO 1999).

Since often mixed infection occurred, it is difficult to assess the effect of ASPV alone (Hadidi et al. 2011). When in combination with ACLSV and ASGV, a negative impact can be seen in production with most commercial cultivars. There are reports of reduced budding success on

pear cultivars (5 to 50%) (Lemoine and Michelesi 1990; Lemoine and Michelesi 1995) and reduced growth of nursery trees (10 to 55%) (Cropley and Posnette 1973; Thomsen 1973)

1.1.4 Transmission and geographical distribution

ACLSV is transmitted by grafting and vegetative propagation (Németh 1986). It is believed that ACLSV is only transmitted by grafting in the field between woody plants (Yaegashi et al. 2011). There is no evidence of vector-, seed- or pollen-borne transmission in any of its hosts (Yoshikawa 2001). In addition, ACLSV can be transmitted by sap inoculation with limited efficiency from woody plants to herbaceous hosts (*N. occidentialis* and *C. quinoa*) (Yaegashi et al. 2011).

ACLSV is one of the most widely distributed viruses of fruit trees (Sutton et al. 2014). It is distributed worldwide and probably present wherever susceptible species grow. For example, an infection rate up to 80-100% in mixed infections with ASGV is reported in China (Wu et al. 1997). A disease incidence ranging from 85% to 90% is reported in Himachal Pradesh, India (Rana et al. 2010). Its infection rates range up to 80-100% in many commercial apple cultivars in USA (Cembali et al. 2003; Németh 1986).

Spread of ASPV is very similar to that of ACLSV. Mixed infections with other apple viruses occur frequently (Sutton et al. 2014). The incidence of ASPV and ACLSV (84.21%) has been reported as the most common mixed infection in apple; the incidence of ASPV together with *Apple mosaic virus* (ApMV) is rare and was only reported in 5.26% of identified mixed infections; the incidence of the mixed infection of ASPV, ASGV and ACLSV is 26.32% (Çağlayan et al. 2006). No vector is known for ASPV.

1.2 Construction of infectious full-length cDNA clones of plant viruses

1.2.1 Historical background

An infectious clone (as cDNAs or as *in vitro*-transcribed RNA copies) of a RNA virus is a plasmid containing the full-length genomic cDNA of the virus. Under control of suitable promoters, the viral genomic cDNA can be transcribed *in vitro* or *in vivo* into the viral genomic RNA, establishing infection in host plants.

Infectious cDNA clones are powerful tools for investigating plant viruses. They can be applied to basic studies of viral life cycles and virus-host interactions, and serve as pools of viral genes for the design of antiviral strategies and for the development of viral vectors (Boyer and Haenni 1994; Nagyová and Subr 2007).

The first infectious clone of a plant RNA virus, *Brome mosaic virus* (BMV), was obtained in 1984 (Ahlquist et al. 1984). Since then, clones to a wide range of different virus species belonging to several virus families such as *Alphaflexiviridae, Betaflexiviridae, Bromoviridae, Closteroviridae, Luteoviridae, Potyviridae* and *Tymoviridae* have been produced, such as *Tobacco mosaic virus* (TMV) (Dawson et al. 1986), *Cucumber mosaic virus* (CMV) (Rizzo and Palukaitis 1990), *Plum pox virus* (PPV) (Riechmann et al. 1990), *Potato virus X* (PVX) (Hemenway et al. 1990), *Barley yellow dwarf virus* (BYDV) (Young et al. 1991), *Potato virus Y* (PVY) (Jakab et al. 1997), *Citrus tristeza virus* (CTV) (Satyanarayana et al. 2001), *Papaya leaf distortion mosaic virus* (PLDMV) (Tuo et al. 2015), *Tomato blistering mosaic virus* (ToBMV) (Blawid and Nagata 2015) and *Maize rayado fino virus* (MRFV) (Edwards et al. 2015). The first infectious clone of ACLSV (isolate P-205) was constructed in 1999 (Satoh et al. 1999). An infectious cDNA clone of ASPV was constructed by Arntjen and Jelkmann (2009).

1.2.2 Methodology

The nature of constructing full-length cDNA clones of viruses is a process of precisely assembling single or multiple DNA fragments of viral genomic cDNAs and vectors. The technique of precise assembly of specific DNA fragments is a critical step in molecular biology. A number of methods for the assembly have been developed, serving various purposes in research.

Given the properties of manipulated DNA fragments, these methods can be grouped roughly into three categories. As one of the simplest and most efficient method, TA cloning has been widely used for cloning (Yao et al. 2016). This method is based on the additional single thymine (T) residue at each end of DNA fragments added by some *Taq* DNA polymerases during polymerase chain reaction (PCR). This method is used rarely in the construction of full-length cDNA clones of plant viruses. But it is reported that an infectious clone of *Muscovy duck parvovirus*, having a ssDNA genome of ca. 5.1 kb, is obtained by TA cloning (Yen et al. 2015). A widely used method to generate infectious clones is traditional cloning method, using restriction enzymes and ligation of DNA *in vitro*. For example, the first

infectious clone of plant RNA viruses, BMV, is constructed using this method (Ahlquist et al. 1984). More recently introduced methods are ligation-independent cloning methods. These methods rely on the generation of complementary overhangs by DNA polymerase, without requiring specific restriction sites or ligation. Several such methods have been developed, including sequence and ligation-independent cloning (Li and Elledge 2012), polymerase incomplete primer extension cloning (Klock et al. 2008), overlap extension cloning (Bryksin and Matsumura 2010), circular polymerase extension cloning (CPEC) (Stevenson et al. 2013) and an *in vivo* yeast homologous recombination (Youssef et al. 2011a). Commercial kits are also available, such as Gibson assembly (Gibson et al. 2010; Gibson et al. 2009) and In-Fusion cloning (Takara Bio Europe, France).

1.2.3 Difficulties in construction of full-length cDNA clones for viruses from woody plants

There are several challenges in construction of full-length cDNA clones of viruses of woody plants (MacKenzie et al. 1997; Meng et al. 2013). First, the titer of viruses is low in woody hosts, making it difficult to extract high concentration viral genomic RNA. Second, the length of the viral genomic cDNA for some viruses (ca. 7.5 kb of ACLSV and 9.5 kb of ASPV) makes one-step cloning poorly efficient. Third, the presence of inhibitory factors (such as polyphenolic compounds or polysaccharides) in fruit trees results in poor quality of viral products in PCR amplification. In order to overcome these difficulties the techniques of CPEC, Gibson assembly and In-Fusion cloning were applied to construction of cDNA clones of the two apple viruses.

The three methods (CPEC, Gibson assembly and In-Fusion cloning) work on fragments having homologous ends, but the mechanism of CPEC is different from that of Gibson assembly and In-Fusion cloning. The CEPC method relies entirely on the polymerase extension mechanism, extending overlapping regions between the insert and vector fragments to form a complete circular plasmid (Stevenson et al. 2013). Gibson assembly uses three enzymes together in a single reaction: the T5 exonuclease removes nucleotides (15 nt) at the 5' termini of the target DNA fragments, creating single-stranded overhangs at 3' termini. The DNA polymerase then fills in the gaps and the DNA ligase seals the nicks (Gibson et al. 2010; Gibson et al. 2009). The In-Fusion cloning is similar to Gibson assembly.

1.3 Inoculation of woody plants with viruses

1.3.1 Methods of Transmission of plant viruses

Plant viruses can be transmitted from plants to plants in different ways. Modes of transmission include mechanical inoculation with sap containing virus particles or viral nucleic acids, vegetative propagation (by bulbs, corms, rhizomes, tubers and runners), through seed, pollen, root, dodder and human behaviors (such as grafting and pruning) and by specific insects, mites, nematodes, and fungi (Agrios 2005).

In addition, molecular biological technologies have been developed to induce virus infections, couples of new methods of transmission of viruses have been invented. These include agroinoculation (or agroinfection) methods (Grimsley et al. 1986; Turpen et al. 1993) and physical methods of electroporation, microinjection, and biolistic inoculation (see for review Rivera et al. 2012). For agroinoculation several techniques are used such as syringe infiltration (Turpen et al. 1993), agrodrench (Ryu et al. 2004) and vacuum infiltration (Rossi et al. 1993).

Among these methods, mechanical inoculation is commonly used, but for those viruses that are not mechanically transmissible, other methods such as agroinoculation, the use of the insect vectors, or grafting may be used. Agroinoculation has become a preferred delivery tool for a variety of viral genomes of interest via expression in plants through *Agrobacterium* binary vectors (Vaghchhipawala et al. 2011).

1.3.2 Difficulties of transmission of viruses to woody plants

It is difficult to transmit viruses to woody fruit trees and grapevine, by conventional mechanical inoculation methods (Yamagishi et al. 2010). This is independent if the inocula are viral particles, nucleic acids or infectious clones. For example, the *Apple latent spherical virus* (ALSV) is transmitted to apple trees in poor infection efficiency (Ito et al. 1992; Li et al. 2004). *Grapevine virus B* (GVB) is transmitted by sap inoculation to hosts with great difficulty (Boscia et al. 1993; Conti et al. 1980). PVX is mechanically transmitted to several citrus species with difficulty (Holmes 1959). The infectious transcripts of ACLSV failed to be transmitted to peach by stem slashing (Youssef et al. 2011a).

Other inoculation methods have been applied to woody hosts, such as biolistic inoculation, syringe infiltration and agrodrench. For example, total RNAs, extracted from ALSV- or ACLSV- infected *C. quinoa* leaves, established infection in apple seedlings with high

efficiency by particle bombardment (Yamagishi et al. 2010). A full-genome cDNA clone of *Citrus leaf blotch virus* (CLBV) was inoculated to Etrog citron plants in high efficiency by syringe infiltration (Vives et al. 2008). Full-length cDNA clones of *Grapevine rupestris stem pitting-associated virus* (GRSPaV) and *Grapevine virus A* (GVA) were introduced to *Vitis vinifera* plantlets by modified agrodrench (Meng et al. 2013; Muruganantham et al. 2009).

Transmission of viruses to woody plants by conventional mechanical inoculation and with molecular methods has always been laborious and time-consuming. None of the current methods will fit all types of viruses and their woody hosts. For example, an infectious cDNA clone of GVA was inoculated by agroinfiltration to *Vitis vinifera* with great difficulty (Muruganantham et al. 2009), while this method was effectively used for CLBV (Vives et al. 2008). CTV can be effectively transmitted to Etrog citron by cutting or slashing the plant stem with a contaminated blade (Garnsey et al. 1977), whilst ALSV cannot be transmitted using this method (Yamagishi et al. 2010).

Thus, it is necessary to establish effective inoculation methods available to different viruses and hosts. In this study, a highly efficient method of agroinoculation of full-length cDNA clones of apple viruses to apple seedlings by vacuum infiltration was established.

1.4 Utilization of the plant viral vectors

Plant viral vectors are constructed for various purposes: to mark viruses for visualization of virus movement and distribution in plants; to express foreign genes in plants; and to examine the relationship between specific genes and plant phenotypes by silencing (Dawson 2014).

1.4.1 The movement of ACLSV in plants

The incorporation of non destructive fluorescent proteins (e.g. the green fluorescent protein, GFP) into viral vectors has allowed an exponential progress on the knowledge of movement of plant viruses. About 75% of reports about transport of plant viruses have been published after the first use of the jellyfish GFP in plant virology (Pallás et al. 2011).

The protein of ca. 50 kDa encoded by ORF2 is suggested to be a MP of ACLSV (German et al. 1990; Sato et al. 1993; Sato et al. 1995). The MP performs multiple functions in host cells, such as increasing the plasmodesmatal size exclusion limit and inducing tubules in infected protoplasts (Satoh et al. 2000). An excellent review on the multiple functions of the MP has been published (Isogai et al. 2007).

To examine the localization, subcellular distribution and cell-to-cell trafficking, the MP of ACLSV P-205 has been expressed. The C-terminus of the MP is fused to the N-terminus of GFP and, depending on purposes, expressed in plants or mesophyll protoplasts of *C. quinoa* and *N. occidentalis*. The results of fluorescence and confocal laser scanning microscopy indicate that the MP of ACLSV targets to plasmodesmata, is distributed as small irregular spots or a fibrous network structure on the periphery of epidermal cells and protoplasts, accumulates in sieve elements in plants, and induces formation of tubular structures on the surface of protoplasts (Satoh et al. 2000; Yoshikawa et al. 1999).

1.4.2 Development of plant viral vectors

Plant viral vectors have been developed as promising alternatives to other methods of gene expression, such as the use of stably transformed transgenic or transplastomic plants, because of some obvious anticipated advantages. For example, compared with the stable nuclear genetic transformation, the transient expression utilizing viral vectors allows for a more efficient, versatile, controlled and safe process (Gleba and Giritch 2011). In addition, viral vectors have anticipated advantages such as speed of expression, high yield, reduced costs and duration of research and development, and extremely high throughput (Gleba et al. 2004; Gleba and Giritch 2011).

So far most viral vectors have been modified from RNA viruses, such as TMV, PVX, *Alfalfa mosaic virus* (AMV), CTV and *Cowpea mosaic virus* (CPMV) (Dawson 2014; Gleba and Giritch 2011).

Different strategies have been used in viral vector development. The vectors function as viruses with extra genes. For example, in construction of the first generation of add-a-gene vectors based on TMV, all of the viral genes needed for replication and movement were kept (Dawson et al. 1989), and the foreign ORF of chloramphenicol acetyltransferase was engineered into TMV either before or after the CP gene under the control by an additional CP subgenomic RNA promoter. Advanced vectors are produced utilizing the properties of deconstructed viruses. For example, expression vectors were designed with a duplication of the three pseudoknots of the TMV 3' nontranslated RNA internally between the foreign ORF and the CP gene (Shivprasad et al. 1999). A series of research suggests that proximity of the gene to the pseudoknots is a key to increase protein expression, see for review (Dawson 2014).

1.5 Aims of this thesis

In this thesis, we aimed to 1) Construction of full-length cDNA clones of ACLSV and ASPV; 2) Optimizing the protocol of constructing full-length cDNA clones of RNA viruses of woody plants; 3) Develop an economic and high-efficiency protocol available for agroinoculation of apple seedlings; 4) Explore and create viral vectors based on the obtained infectious full-length cDNA clones of ACLSV to study ACLSV systemic movement in host plants and to express foreign proteins in plants.

2. Materials and methods

2.1 Materials

2.1.1 Virus source

Four different virus isolates were used in the present work. All isolates are kept under glasshouse or field conditions at the Julius Kuehn Institute (JKI), Dossenheim: *Apple chlorotic leaf spot virus* (ACLSV) isolate (27)/85 in peach; ACLSV isolate 38/85 in apple; ACLSV isolate (36)/88 in pear; *Apple stem pitting virus* (ASPV) isolate 40/87 in apple.

2.1.2 Plant materials

Herbaceous and woody plants were used as test or host plants in this work. All plants were produced in JKI, Dossenheim. These plants were virus-free. The herbaceous plants were *Nicotiana occidentalis* 37B and *Chenopodium quinoa.* They were germinated and grown in the glasshouse of JKI. Growing conditions of the glasshouse were 20-26°C and 50-60% humidity with daily luminous flux from 120 to 560 kilolux. Unless mentioned otherwise four to six-leaf-stage *N. occidentalis* 37B and four to six-leaf stage *C. quinoa* were the test-plants.

Woody plants contained one to three-month seedlings of *Malus domesticus* cv. Golden Delicious, and one-year seedlings of G. Delicious, *Prunus persica* and *P. armeniaca*. The one to three-month G. Delicious were germinated from seeds in sterile sand in darkness at 4°C (refrigerator). They were maintained and grown in the refrigerator until use. The one-year-old G. Delicious, *P. persica* and *P. armeniaca* were kept in screen house in JKI.

2.1.3 Bacterial strains and plasmids

Table 2.1 Bacterial strains of *Escherichia coli* and *Agrobacterium tumefaciens*

Bacterial strain	Application	Source
E. coli NM 522	Electro competent cells	Lab stored
E. coli NEB® 5-alpha (High Efficiency)	Chemically competent cells	New England Biolabs GmbH, Germany
E. coli NEB® 10-beta (High	Chemically competent cells	New England Biolabs

Bacterial strain	Application	Source
Efficiency)		GmbH, Germany
E. coli Steller™	Chemically competent cells	Takara Bio Europe, France
A. tumefaciens strain ATHV	Electro competent cells	Lab stored
A. tumefaciens strain GV2260	Electro competent cells	Lab stored

Table 2.2 Plasmids used in labeling of ACLSV

Name	Application	Obtained from	References
pV297	Binary vector modified from pCB301	E. Maiss	(Xiang et al. 1999)
pDoc-G	Donor of Ermald GFP	A. Wensing	(Lee et al. 2009)
pK2GW7	Donor of mCherry	Borja Garnelo-Goméz	(Karimi et al. 2002)
p2488	Donor of iLov	E. Maiss	-
pIF3-15	Infectious clone of ACLSV 38/85A	Cloned during this study	section 2.2.2.3 and section 3.1.2
pIF3-19	Infectious clone of ACLSV 38/85B	Cloned during this study	section 2.2.2.3 and section 3.1.2
pIF13-9	Label MP at C-terminus with GFP	Cloned during this study	section 2.2.2.3 and section 3.5.1
pIF18-2	Label MP at C-terminus with GFP	Cloned during this study	section 2.2.2.3 and section 3.5.1
pIF25-7	Label MP at C-terminus with GFP	Cloned during this study	section 2.2.2.3 and section 3.5.1
pG11-15	Label CP at N-terminus with mCherry	Cloned during this study	section 2.2.2.3 and section 3.5.1
pIF24-6	Label CP at N-terminus with mCherry	Cloned during this study	section 2.2.2.3 and section 3.5.1
pIF23-1	Label CP at N-terminus with mCherry	Cloned during this study	section 2.2.2.3 and section 3.5.1
pIF16-1	Label CP at C-terminus with mCherry	Cloned during this study	section 2.2.2.3 and section 3.5.1
pIF20-16	A linker of $(EAAAK)_4$ between CP and mCherry	Cloned during this study	section 2.2.2.3 and section 3.5.1
pIF27-10	Label CP at C-terminus with iLov	Cloned during this study	section 2.2.2.3 and section 3.5.1

2.1.4 Molecular biological reagents

Table 2.3 Oligonucleotides used in the present work

Reference no. in text	Primer names	Tm (°C)	Sequence*
#001	ZL-ACLSV-F	51	TGATACTGATACAGTGTACACTCAC
#002	ZL-ACLSV-R	51	GTAGTAAAATATTTAAAAGTCTACAGG
#003	297exACLSV-IF-F	55	**taaatattttactacGGGTCGGCATGGCATCTC

Reference no. in text	Primer names	Tm (°C)	Sequence*
#004	297exACLSV-IF-R	55	actgtatcagtatcaCCTCTCCAAATGAAATGAAC
#005	ZL-ASPV-F	55	GGATACGCAAACAAACTCTGAA
#006	ZL-ASPV-R	55	GAAAATCTAGTTAAAACAAAAATAAG
#007	ZL-pV297 ex ASPV2-F	55	tttaactagattttcGGGTCGGCATGGCATCTC
#008	ZL-pV297 ex ASPV2-R	55	tttgtttgcgtatccCCTCTCCAAATGAAATGAACTTC CTTATATAG
#009	ZL140813-f-01	52	AACGCTCTTTTCTCTTAGGT
#010	ZL140813-r-02	52	GAGGCGTTACGTCAATCTGT
#011	ZL140814-f-03	48	GGTATTTAATTGGAGTGTTT
#012	ZL140814-r-04	48	TACAAATACAAATACATACTAAGG
#013	ZL-ASPV-05-F	45	TAAAGGAAAGGCTATCGTT
#014	ZL-ASPV-06-R	45	CGGTAGGAGTGGGGGCTGAGGT
#015	ZL-ASPV-07-F	53	CTCATGCTGCAAACTCAAAGTC
#016	ZL-ASPV-08-R	53	TCCCTTAGCCATCCGAGTG
#027	ZL-ATHV10-G15-F	60	TGAGGCGTTCGCCCCTGA
#028	ZL-ATHV10-G16-R	60	CATACTTGGCGGAAAGTCATG
#029	ZL-ATHV10-G17-F	60	tttccgccaagtatgAGCAAGGGCGAGGAGCTG
#030	ZL-ATHV10-G18-R	60	cttcttgcccatcatTTACTTGTACAGCTCGTCCATG
#031	ZL-ATHV10-G19-F	52	gagctgtacaagtaaATGATGGGCAAGAAGAAAG
#032	ZL-ATHV10-G20-R	52	ggggcgaacgcctcaCATACTTGGCGGAAAGTC
#036	ZL-ATHV10-G24-R	60	ggggcgaacgcctcaTTACTTGTACAGCTCGTCCAT G
#037	ZL-ATHV10-G25-R	60	TTACTTGTACAGCTCGTCCATG
#038	ZL-ATHV10-G26-F	51	AGCAAGGGCGAGGAGCTG
#046	ACLSV-FP-6860	55~60	TTCATGGAAAGACAGGGGCAA
#047	ACLSV-RP-7507	55~60	AAGTCTACAGGCTATTTATTATAAGTCTAA
#048	ASPV-F-8869	55	ATGTCTGGAACCTCATGCTGCAA
#049	ASPV-R-9211	55	TTGGGATCAACTTTACTAAAAAGCATAA
#050	IF3-19-nurMP-F	55	ACCTGATGATCCATTGGAATG
#054	ZL-ATHV10-m01-F	52	TAATCTGATGAAGAGGTTTGG
#055	ZL-ATHV10-m02-R	52	AACGCAAAGATCAGTCGTAAC
#056	ZL-ATHV10-m03-F	55	actgatctttgcgttATGGTGAGCAAGGGCGAG
#057	ZL-ATHV10-m04-R	55	ctcttcatcagattaCTACTTGTACAGCTCGTCC
#059	ZL-ATHV10-m08-R	50	ctcttcatcagattaAACGCAAAGATCAGTCGTAAC
#060	ZL-ATHV10-m09-R	51~56	CTACTTGTACAGCTCGTCCATGC
#061	ZL-ATHV10-m10-F	51~56	ATGGTGAGCAAGGGCG
#064	ZL-ATHV10-m11-F	58	ATGGCGGCAGTGTTGAACC
#065	ZL-ATHV10-m12-R	58	CCTTCGCCTTCTGATCTTGTC
#066	ZL-ATHV10-m13-F	56	atcagaaggcgaaggATGGTGAGCAAGGGCGAG
#067	ZL-ATHV10-m14-R	56	caacactgccgccatCTTGTACAGCTCGTCCATGC
#068	ZL-ATHV10-m15-F	50	ATGATGGGCAAGAAGAAAG
#071	ZL-ATHV10-m18-R	50	cttcttgcccatcatTCACATACTTGGCGGAAAG
#080	ZL-ATHV10-mp636-F	52	CAAGGGAGCATGAGATACC
#083	ZL-ATHV10-i01-F	55	TAATCTGATGAAGAGGTTTGG
#084	ZL-ATHV10-i02-R	55	AACGCAAAGATCAGTCGTAAC

Reference no. in text	Primer names	Tm (°C)	Sequence*
#085	ZL-ATHV10-i03-F	52	actgatctttgcgttATAACAATGATAGAGAAGAATTTCG
#086	ZL-ATHV10-i04-R	52	ctcttcatcagattaTACATGATCACTTCCATCG
#087	ZL-Re-01-F	53	TCTGATGAAGAGGTTTGGTTC
#088	ZL-Re-02-R	53	TTACTTGTACAGCTCGTCCA
#090	ZL-Re-03p-F	56	gagctgtacaagtaaCGAAATCCATTACTTCAGAG
#091	ZL-Re-04-R	56	aacctcttcatcagaCTAAATGCAAAGATCAGTTGTAAC
#093	ZL-Re-06-R	55	TCACACACCTGGCGGAAAG
#094	ZL-Re-07-F	51	ccgccaggtgtgtgaATGATGGGCAAGAAGAAAGTC
#096	ZL-ATHV9-MP1050-F	54	AAGGAGGATGGCAGCAGTTC
#097	ZL-ATHV10-CP-mCh-R	54	CTGCCGCCATCTTGTACAGC
#098	ZL-ATHV9-CP-R	51	CTTACTTCCTACTTCCGGCATG
#099	ZL-ATHV10-mp636-F	49	CAAGGGAGCATGAGATACC
#100	ZL-Re-09-F	58	cagaatctattctgaTATCAGCCCTTTCAAAAAGGC
#101	ZL-Re-10-R	58	cttcttgcccatcatTCACACACCTGGCGGAAAG
#102	ZL-Re-11-F	53	ATGATGGGCAAGAAGAAAGTC
#103	ZL-Re-12-R	53	TCAGAATAGATTCTGGAGCTTTTCACC
#104	ZL-Re-13-F	62	cagaatctattctgaCATAAGCCCCTTCAAGAGGG
#105	ZL-Re-14-R	62	gaaatctctctgactTTACTTGTACAGCTCGTCCATGC
#106	ZL-Re-15-F	52	AGTCAGAGAGATTTCCCTGG
#107	ZL-Re-16-R	52	TCAGAATAGATTCTGGAGC
#108	Oligo$(dT)_{18}$	-	TTTTTTTTTTTTTTTTT
#109	M13	49	CACGACGTTGTAAAACGAC
#110	seq-r-04	49	GACCTTTTGTTGGGCCTTATTCAT
#111	Seq-f	55	GAGGAGCATCGTGGAAAAAGAAG
#112	Seq-r	55	GACTGGTGATTTTTGCGGACTCT

* Unless mentioned otherwise the applied concentration of each oligonucleotide was 10 µM.

** Small letters of 15 nts indicate the nucleotides additional to their templates

Table 2.4 Standard markers

Marker	Company
GeneRuler™1 kb plus DNA ladder	Thermo Scientific
PageRuler™ prestained protein ladder	Thermo Scientific

Table 2.5 Enzymes and enzyme-based kits

Enzyme	Concentration	Company
FastDigest *Dpn*I	-*	Thermo scientific
FastDigest *Xba*I	-	Thermo scientific
RevertAid Premium reverse transcriptase	200 U/µl	Thermo Scientific

Enzyme	Concentration	Company
ReverseAid reverse transcriptase	200 U/µl	Thermo Scientific
KAPA Taq DNA polymerase	5 U/µl	Kapa Biosystems, US
PRECISOR high-fidelity DNA polymerase	2 U/µl	BioCat GmbH, Germany
Gibson Assembly® master mix	2×	New England Biolabs GmbH, Germany.
In-Fusion® HD enzyme premix	5×	Clontech, Takara Bio Europe, France
T4 DNA ligase (pGEM®-T easy vector system)	3 U/µl	Promega GmbH, Germany

*Concentration is not provided by manufacturer

Table 2.6 Primary and secondary antibodies used in western blot

Name	Antigen / Conjugate	Source	Dilution	Reference / Company
Anti-mCherry	Red fluorescent protein	Rabit, polyclonal (IgG)	1:1000	US Biological life sciences
Anti-CP	ACLSV CP		1:2000	Kind gift from N. Yoshikawa
Anti-50K	ACLSV MP		1:2000	(Sato et al. 1995; Yoshikawa and Takahashi 1989)
Anti-Rabit IgG	Alkaline phosphotase	Goat	1:1000	Sigma-Aldrich Chemie GmbH, Germany

Table 2.7 Silica-based kits

Name	Company
RNeasy® plant mini kit	QIAGEN GmbH, Germany
QIAquick® PCR purification kit	QIAGEN GmbH, Germany
QIAquick® gel extraction kit	QIAGEN GmbH, Germany
QIAEX® II gel extraction kit	QIAGEN GmbH, Germany
QIAprep® spin miniprep kit	QIAGEN GmbH, Germany

Table 2.8 Buffers and chemicals

Buffer / Solution / Reagents	Utilities	Composition / Company
Grinding buffer	Silica capture	6 M guanidine hydrochlorid, 0.2 M NaOAc, 25 mM EDTA, 1.0 M KOAc, 2.5% (w/v) PVP40 and H_2O
NLS buffer	Silica capture	10 g N-Lauroylsarcosine sodium salt, 100 ml distilled H_2O, optional 5% β-mercaptoethanol
1× Silica capture buffer	Silica capture	150 µl ethanol, 300 µl 6 M NaI and 25 µl resuspended silica
NaI solution	Silica capture	0.75 g Na_2S0_3 in 40 ml Milli-Q H_2O and 36 g NaI

Buffer / Solution / Reagents	Utilities	Composition / Company
Resuspended silica	Silica capture	1 g silicon dioxide in 1 ml Milli-Q H_2O, pH 2.0
Wash buffer	Silica capture	10 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 50.0 mM NaCl, 50% (v/v) ethanol
1× TAE buffer	Electrophoresis	40 mM Tris-acetate, 1 mM EDTA
DNA loading dye (6x orange)	Electrophoresis	50% (v/v) glycerol, 0.25% (w/v) Orange G and Mili-Q H_2O
DNA loading dye (6x blue)	Electrophoresis	Thermo Scientific
SOC medium	Transformation	2% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.05% (w/v) NaCl, 2.5 mM KCl, 10 mM $MgCl_2$ and 20 mM glucose
LB liquid	Culture medium	1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl and ddH_2O; pH 7.0
LB solid	Culture medium	LB media liquid, 1.5% (w/v) agar
LB glycerol buffer	Store medium	LB media liquid, 50% (v/v) glycerol
YEB liquid	Culture medium	0.5% (w/v) nutrient broth, 0.5% (w/v) bacto tryptone, 0.1% (w/v) yeast extract and 0.5% (w/v) sucrose; pH 7.4
YEB solid	Culture medium	YEB liquid, 1.5% (w/v) agar
GSM buffer	Store medium	50% (v/v) glycerol, 100 mM $MgSO_4$ and 25 mM Tris pH 7.5
Herb-herb inoculation buffer	Mechanical inoculation	0.07 M phosphate buffer, 0.01 M sodium diethyldithiocarbamate trihydrate and 0.02 M sodium thioglycolate, pH 7.0
Woody-herb inoculation buffer	Mechanical inoculation	0.05 M sodium phosphate buffer, 0.02 M sodium diethyldithiocarbamate trihydrate, 0.04 M sodium thioglycolate and 2.5% (v/v) nicotin, pH 7.0
1× Protein extraction buffer	Western blot	100 mM Tris (pH 8.0), 100 mM NaCl, 5 mM EDTA and 0.5% (v/v) Tween 20, 20 mM DTT and Mili-Q H_2O
1× Protein loading buffer	Western blot	1% (v/v) SDS and 0.025 mg/ml bromophenol blue
SDS-PAGE running buffer	Western blot	23 mM Tris (pH 8.0), 190 mM glycine, 0.1% (w/v) SDS
1× Transfer buffer	Western blot	23 mM Tris (pH 8.0), 190 mM glycine, 10% (v/v) methanol
1× PBS buffer	Western blot	137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4 and Mili-Q H_2O
1× PBS-T buffer	Western blot	1 × PBS buffer, 0.1% (v/v) Tween 20

Buffer / Solution / Reagents	Utilities	Composition / Company
Blocking buffer	Western blot	1 × PBS-T buffer, 5% (w/v) milk powder
AP buffer	Western blot	5 mM $MgCl_2$, 100 mM NaCl and 100 mM Tris (pH 9.6)
Silicon dioxide	Silica capture	99%, 0.5-10 µm (approx. 80% between 1~5 µm), Sigma-Aldrich Co. LLC.
Universial Agrose	Electrophoresis	Bio&SELL GmbH, Germany
H_2O HPLC grade*	-	PanReac AppliChem, Illinois Tool Works Inc.
Mili-Q H_2O	-	Lab produce
dNTPs	-	Fermentas
Midori Green advanced DNA stain	Electrophoresis	Nippon Genetics Europe GmbH, Germany
Skim milk powder	Western blot	Merck KGaA, Germany
BCIP/NBT	Western blot	SERVA Electrophoresis GmbH, Germany
Rotiphorese® Gel40 (37.5:1)	SDS-PAGE	Carl Roth GmbH, Germany
Mikrozid® AF liquid	Disinfection	Schülke & Mayr GmbH, Germany
antifect® N liquid	Disinfection	Schülke & Mayr GmbH, Germany
Maywax	Graft	Hermann Meyer, Germany
Carborundum (600 mesh)	Mechanical inoculation	-

* Unless mention otherwise H_2O referred to H_2O HPLC grade in the present work.

2.1.5 Apparatus, consumable and software

Table 2.9 Apparatus

Equipment	Type	Company
Benchtop fluorometer	Invitrogen™ Qubit® 2.0	Thermo Scientific
PCR cycler	Mastercycler® personal	Eppendorf AG, Germany
PCR cycler	GeneTouch thermal cycle	Bioer, China
Benchtop centrifuge	Heraeus Biofuge Fresco	DJB Labcare, UK
Benchtop centrifuge	Centrifuge 5804	Eppendorf AG, Germany
Water bath TW12	Thermo Haake® DC10-P14	Sigma-Aldrich Chemie GmbH, Germany
UV transilluminator	Reprostar 3	CAMAG, Germany,
Incubator shaker	Innova 4430	GMI Inc., USA
Heating block	Thermomixer comfort	Eppendorf AG, Germany
Electrophoresis power supply A	PowerPac 300	Bio-Rad Laboratories GmbH, Germany
Electrophoresis cells	Wide Mini-Sub® Cell GT	Bio-Rad Laboratories GmbH, Germany
Horizontal agarose gel	Sub-Cell GT systems	Bio-Rad Laboratories GmbH,

Equipment	Type	Company
caster		Germany
Vacuum pump	Büchi® V-500	Sigma-Aldrich Chemie GmbH, Germany
Vacuum controller	Büchi® B-721	Sigma-Aldrich Chemie GmbH, Germany
Dessicator	-	Glaswerk Wertheim, Germany
High-Speed centrifuge	Avanti® J-26S XP	Beckman Coulter GmbH, Germany
Centrifuge rotor	JA-25.50	Beckman Coulter GmbH, Germany
Centrifuge rotor	JA-14	Beckman Coulter GmbH, Germany
Centrifuge rotor	JS-5.3	Beckman Coulter GmbH, Germany
Electrophoresis Power Supply	PS9009	Gibco BRL
Electrophoresis Power Supply	Consort E844	Sigma-Aldrich GmbH, Germany
Electrophoretic Transfer system	Mini Trans-Blot®	Bio-Rad Laboratories GmbH, Germany
Vertical Electrophoresis System	SE250	Hofer Inc., USA
Dual gel caster	SE245	Hofer Inc., USA
Biological safety cabinets	SterilGARD® Hood, Class II, Type A/B3	Baker Company Inc, USA
Rocking Shaker	Duomax 1030	Heidolph Instruments GmbH, Germany
Homogenizer Hand Model	-	BIOREBA AG, Switzerland
Sterilized needles	-	-
Mortars and pestles	-	-

Table 2.10 Consumables

Experimental consumable	Company
Extraction bag Universal	BIOREBA AG, Switzerland
Hybond® ECL® Nitrocellulose membrane	GE Healthcare UK Limited, UK
Immobilon®-P transfer membrane	Milipore Corporation, USA
Filter tips	Neptune Scientific
Parafilm M	Singma-Aldrich GmbH, Germany
Rubber (Fleicoband ‘A’ 240 × 60 mm)	Hermann Meyer, Germany
Petri dishes	Greiner Bio-One GmbH, Germany
Semi-micro cuvette	Sarstedt AG & Co., Germany
Mix2Seq Kit	Eurofins Genomics, Germany

Table 2.11 *In silico* tools

Software	Company / Reference
MEGA5	(Tamura et al. 2011)
Lasergene 7.1.0	DNASTAR, Inc.
NCBI tools	https://www.ncbi.nlm.nih.gov/
CLC Main Workbench 7.8.1	QIAGEN Aarhus A/S

2.2 Methods

2.2.1 Molecular biology

2.2.1.1 Total nucleic acids extraction

To extract the total nucleic acids from plant materials, two methods were used in the present work, i.e. silica capture and RNeasy plant mini kit (Table 2.7). The two methods served different purposes.

For the purpose of diagnosis of viral infection, the silica capture was used following the protocol by Rott and Jelkmann (2001) with modification. Fresh leaf blades of ca. 300 mg were ground in 3 ml grinding buffer (Table 2.8) in a universal extraction bag (Table 2.10). The homogenized plant material of 500 µl was then transferred to 100 µl NLS buffer (Table 2.8) to a 1.5 ml reaction tube. The mixture was incubated in a heating block at 70°C with intermittent shaking (300 rpm) for 10 min, followed by cooling on ice for 5 min. By centrifugation at 13,000 rpm for 10 min, the residual plant materials were separated from the mixture. The supernatant of 300 µl was pipetted to 1× silica capture buffer (Table 2.8) to a new tube. This silica mixture was incubated at room temperature for 10 min with shaking. The silica particles were collected by centrifugation at 6,000 rpm for 30 sec. They were washed twice by resuspension in 500 µl wash buffer (Table 2.8). After that, the pellet of silica particles was shortly centrifuged to pipette the residual wash buffer, followed by air-drying in a biological safety cabinet for several minutes. The dry pellet was resuspended in 150 µl H_2O and incubated at 70 °C for 5 min with intermittent shaking. Through separating the silica particles by centrifugation at 13,000 rpm for 3 min, the nucleic acids solution was transferred to a new tube and stored at -20°C.

For the purpose of producing full-length genomic cDNAs of viruses, the RNeasy plant mini kit was used for RNA extraction. To obtain optimal RNA yield and purity, the amount of 100

mg plant material was processed. Buffer RLT (see the RNeasy mini handbook for more information) was the lysis buffer of choice in the present work.

The concentration of the RNAs was measured using a Qubit 2.0 fluorometer (Table 2.9) according to the manufacturer's instructions. The RNAs were stored at -20°C until use.

2.2.1.2 Reverse transcription polymerase chain reaction (RT-PCR)

The cDNAs of viruses were synthesized from total nucleic acids using reverse transcriptase (RTase). Two types of RTases were used in the present work, i.e. RevertAid RTase and RevertAid Premium RTase (Table 2.5). Typically for diagnosis of viral infection, the ReverseAid RTase was the enzyme of choice. To produce the full-length genomic cDNAs of viruses, the RevertAid PRTase was used. Primer #108 was always the primer for RT.

The reaction mixture and cycling conditions of RT using ReverseAid RTase and PRTase were shown below in Table 2.12 and Table 2.13, respectively.

Table 2.12 The reaction mixture and cycling conditions of RT-PCR

Step	Components	Volume (µl)	Cycling conditions
1	Silica-captured RNAs	X*	Incubate the step 1 mixture at 70°C for 10 min
	Primer #108	1	
	H_2O	5.75-X	
2	5× Reaction Buffer	2	Add step 2 components and incubate the total mixture at 42°C for 50 min followed by 70°C 10 min
	10 mM dNTPs	1	
	RTase	0,5	

* The volume ≤ 5.75 µl. The amount of RNA was around 100 to 200 ng.

Table 2.13 The reaction mixture and cycling conditions of PRT-PCR

Step	Components	Volume (µl)	Cycling conditions
1	RNeasy-extracted RNAs	4	Incubate the step 1 mixture at 65°C for 10 min
	Primer #108	1	
	10 mM dNTPs	1	
	H_2O	8.5	
2	5× RT Buffer	4	Add step components and incubate the total mixture at 50°C for 30 min followed by 85°C 5 min
	RNase Inhibator	0.5	
	PRTase	1	

2.2.1.3 Polymerase chain reaction (PCR)

Two types of polymerases were used in the present work, i.e. KAPA *Taq* DNA polymerase and PRECISOR high-fidelity DNA polymerase (Table 2.5). To detect virus fragments, to perform colony PCR or for TA cloning purpose, KAPA *Taq* DNA polymerase was the enzyme of choice. To amplify fragments with blunt ends, such as full-length fragments of viral genomic cDNAs and linear vectors, PRECISOR high-fidelity DNA polymerase was used in PCRs.

Usually a volume of 12.5 µl of reaction mixture was set in PCRs using KAPA *Taq* DNA polymerase. The reaction mixture and the cycling conditions were shown in Table 2.14 and Table 2.15, respectively. A volume of 50 µl of reaction mixture was set in PCRs using PRECISOR high-fidelity DNA polymerase. The reaction mixture and the cycling conditions were shown in Table 2.16 and Table 2.17, respectively.

Table 2.14 PCR reaction mixture using KAPA *Taq* DNA polymerase

Components	Volume (µl)
10× Buffer A	1.25
10 mM dNTPs	0.25
Primer 1	0.5
Primer 2	0.5
cDNA template*	1.25**
polymerase	0.05
H_2O	8.7

* The templates can also be colonies and bacteria culture. The culture of bacteria was heated at 70°C for 15 min before using as templates.
** In colony PCR, the picked colonies were used directly as templates without additional H_2O filling the volume up to 1.25 µl.

Table 2.15 Cycling conditions using KAPA *Taq* DNA polymerase

Temperature (°C)	Duration	Cycles
95*	3 min	1
95	30 s	
X**	30 s	35
72	1 min/kb	
72	2 min/kb	1***

* In colony PCR the temperature was increased from 95 to 98°C
** The parameters of annealing of primers were listed in the Table 2.3
*** This cycle was an optional step, it was necessary only when preparation of inserts for TA cloning

Table 2.16 PCR reaction mixture using PRECISOR high-fidelity DNA polymerase

Components	Volume (µl)
5× Buffer*	10
2 mM dNTPs	6.25
Primer 1	2
Primer 2	2
Template	X**
Polymerase	1
H_2O	28.75-X

* Two types of buffer were offered with the polymerase. To amplify genomic cDNAs of viruses from cDNA templates, the GC buffer was the choice. To linearize plasmids of < 5 kb, the Hifi buffer was used; to linearize plasmids of > 5 kb, the GC buffer was used. To produce partial fragments < 3 kb from plasmid template, the Hifi buffer was used; otherwise GC buffer was used.

** Usually to amplify genomic cDNAs of viruses, cDNA template of 4 µl was used. To linearize plasmid of < 5 kb, template of plasmid of 0.1-0.5 ng was used; to linearize plasmid of > 5 kb or amplify partial fragments from plasmids, template of plasmid of 20-30 ng was used.

Table 2.17 Cycling conditions using PRECISOR high-fidelity DNA polymerase

Temperature (°C)	Duration	Cycles
98	2 min	1
98°C	30 s	
X*	30 s	25-35***
72°C	15-30 sec/kb**	
72°C	10 min	1

* The annealing temperature of each primer was listed in Table 2.3.
** For cDNA templates, to linearize plasmids and to amplify partial fragments of > 3 kb from plasmid templates, the time was 30 sec/kb. To amplify partial fragments < 3 kb from plasmid templates, the time was 15 sec/kb.
*** The number of cycles was optimized for different assays.

To amplify the short fragment of 90 bp of $(EAAAK)_4$ linker from synthesized oligonucleotides. A 25 µl reaction mixture was prepared: 5 µl 10× Hifi Buffer, 3.25 µl 2 mM dNTPs, 5 µl of primer #075, 5 µl of the Oligonucleotides #076 as template, 0.2 µl PRECISOR high-fidelity DNA polymerase and filled up with H_2O to the final volume. The cycling conditions were 95°C for 2 min, 3 cycles of 50°C for 20 sec and 72°C for 10 sec.

2.2.1.4 Agarose gel electrophoresis

Electrophoresis was conducted in Sub-Cell GT system. The system consisted of power supply PowerPac 300, Wide Mini-Sub Cell GT and horizontal agarose gel casters (Table 2.9). Gels were prepared ahead of the run and supplemented with 20 µl/l Midori Green advanced DNA stain (Table 2.8). Samples were mixed with orange or blue loading dye, and loaded on the gel together with a standard marker of 1 kb plus DNA ladder (Table 2.4) for size determination.

Electrophoresis was performed through 1× TAE buffer (Table 2.8) at 90-110 V for 30-40 min. DNA was visualized on a Reprostar 3 UV transilluminator system (Table 2.9).

The concentration of gels was decided according to the purposes and the size of fragments. In general detection purposes, 1% (w/v) agarose gel was used. To purify fragments by gel extraction, 2% (w/v) agarose gel was used for fragments of ca. 90 to 150 bp. Gels of 1% (w/v) were used for fragments of ca. 200 bp to 8 kb. Gels of 0.7% (w/v) were prepared for fragments of > 8 kb.

2.2.1.5 DNA purification

DNA purification was conducted using different gel extraction kits, i.e. QIAquick and QIAEX II gel extraction kit (Table 2.7). The separation of DNAs in agarose gels were described above, see section 2.2.1.4.

If the size of target DNA fragments was between 90 and 150 bp or > 8 kb, the DNAs in the cut gel were extracted using the QIAEX II gel extraction kit. The extraction was performed according to the manufacturer's protocols.

If the size of the target DNA fragments was between 200 bp and 8 kb, the DNAs were extracted from the cut gels using QIAquick gel extraction Kit. The extraction was conducted according to the manufacturer's protocols.

The concentration of the purified DNAs was measured using a Qubit 2.0 fluorometer (Table 2.9) according to the manufacturer's instructions. The purified DNAs were stored at -20°C for use.

2.2.1.6 Cloning of the target fragments

For fusion of inserts and vectors, three different methods were used in the present work: circular polymerase extension cloning (CPEC) (Quan and Tian 2009), Gibson assembly and In-Fusion cloning (Table 2.5). The three methods achieved cloning based on the homologous ends of inserts and vectors (see section 1.2.3). Before fusion, the inserts and vectors were purified by Gel extraction (sections 2.2.1.4 and 2.2.1.5).

In CPEC assays the PRECISOR high-fidelity DNA polymerase (Table 2.5) was the enzyme of choice. The CPEC reaction mixture (25 µl) and cycling conditions were as below.

Table 2.18 The reaction mixture of the CPEC

Components	Volume (µl)
5× Hifi Buffer	5
2 mM dNTPs	3.25
Polymerase	0.5
Inserts	X*
Vectors	Y*
H_2O**	fill up to 25 µl

* The volumes of inserts and vectors were determined according to their concentration. X+Y ≤ 16.25.
** H_2O was an optional component. The volume could be 0 µl.

Table 2.19 The cycling conditions in the CPEC

Temperature (°C)	Duration	Cycles
98	30 sec	1
98	10 sec	15
55	20 sec	
72	30 sec/kb	

The In-Fusion cloning and Gibson assembly reaction mixture were prepared according to the manufacturer's instructions, in the present work the preparation of each reaction mixture was described for each cloning. The prepared reaction mixtures were incubated at an isothermal condition of 50°C for 1 to 2 hours.

After the fusion step, the assembled product was directly used for transformation of competent *E. coli* cells or stored at -20°C.

2.2.2 Molecular cloning of viruses and viral vectors

2.2.2.1 Construction of full-length cDNA clones

To rescue the viruses of ACLSV and ASPV from their host plants, full-length cDNA clones were constructed. In the present work, the binary vector of pV297 was used as vectors (Table 2.2). It possesses a *Cauliflower mosaic virus* (CaMV) 35S promoter and the hepatitis delta viral ribozyme (HDV) sequence followed by a CaMV 35S polyadenylation (pA) signal (Figure 2.1).

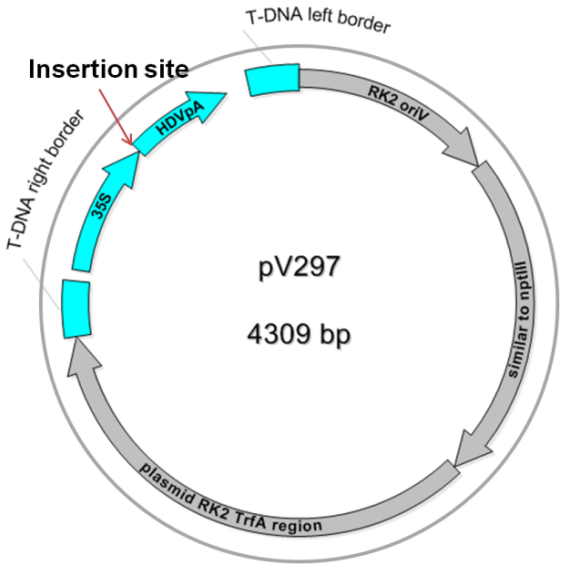

Figure 2.1 The structure of the binary vector pV297. The insertion site of genomic cDNA of viruses was between a CaMV 35S promoter and a HDVpA (indicated by the red arrow).

The plasmid pV297 was linearized by PCR. To produce linear vectors for ACLSV, the primers #003 and #004 were used (Table 2.3). Using this primer pair, extensions of 15 bp were attached to the 5' and 3' ends of the linear vectors. The extensions were homologous to the 3' and 5' ends of genomic cDNAs of ACLSV, respectively. To produce linear vectors for ASPV, the primers #007 and #008 were used (Table 2.3). Linear vectors with extensions homologous to ASPV were generated too. In the PCR assays the enzyme of PRECISOR high-fidelity DNA polymerase was the choice. The reaction mixture and cycling conditions were set according to Table 2.16 and Table 2.17 (see section 2.2.1.3).

The full-length genomic cDNAs of the viruses were amplified from total nucleic acids of infected plants by RT-PCR. The total nucleic acids of infected plant materials were extracted using RNeasy plant mini kit (see section 2.2.1.1). With templates of the total nucleic acids extraction, cDNAs were generated by reverse transcription assays (see section 2.2.1.2). With the templates of cDNAs, genomic cDNAs of viruses were amplified by PCR. To amplify genomic cDNAs of ACLSV, the primers #001 and #002 (Table 2.3) were used. The primers #005 and #006 (Table 2.3) were used for amplification of genomic cDNAs of ASPV. In the PCR assays, the PRECISOR high-fidelity DNA polymerase was used. The reaction mixture and cycling conditions were as in Table 2.16 and Table 2.17 (see section 2.2.1.3).

The fragments of the vectors and the full-length genomic cDNAs of viruses were purified using a gel extraction kit (see section 2.2.1.5). Before purification, the templates of plasmid

pV297 were digested with *Dpn*I. The enzyme of *Dpn*I of 1 µl was added directly to the PCR reaction mixture. This mixture was incubated for 15 min at 37°C.

The gel purified fragments of the vectors and full-length genomic cDNAs were fused using CPEC, Gibson assembly or In-Fusion cloning (see section 2.2.1.6).

The fusion reaction mixture was used for transformation of competent *E. coli* cells by heat shock. Heat shock was as described in section 2.2.3.2.

Positive colonies were selected by colony PCRs (see section 2.2.1.3). For selecting full-length cDNA clones of ACLSV, primer pairs of #009/#010 and #011/#012 were used. Each primer pair encompassed the fusion parts of pV297 and genomic cDNAs of ACLSV. In addition, the primer pair of #046/#047 (Menzel et al. 2002) was used for the selection too. For selecting full-length cDNA clones of ASPV, primer pairs of #048/#049, #013/#014 and #015/#016 were used.

The plasmids of positive colonies were extracted from liquid cultures using QIAprep spin miniprep kit according to the manufacturer's protocol.

The plasmids were delivered into competent *A. tumefaciens* strain ATHV by electroporation (see section 2.2.3.2). Then the transformed *A. tumefaciens* were used for agroinoculation of test-plants (see section 2.2.4.1 and section 2.2.4.3).

2.2.2.2 Strategies of labeling viral proteins with fluorescent proteins

To fuse fluorescent proteins to the proteins of ACLSV, recombinant plasmids containing marker genes, i.e. Emerald-GFP (EmGFP), mCherry and iLov, were constructed. Three different strategies were used for labeling ACLSV proteins.

Strategy A: the overlapping open reading frames of movement protein (MP) and coat protein (CP) genes were selected for insertion of the fluorescent genes. To keep the genes intact, the overlapping part was duplicated. The potential promoter for CP was retained.

Strategy B: as in strategy A the overlapping open reading frames of MP and CP genes were also the insertion site. To keep the gene intact and to avoid the deletion of the marker gene insert caused by homologous recombination, the heterogeneous genes of MP and CP in pIF3-15 and pIF3-19 (Table 2.2) were recombined.

Strategy C: the 3' end of CP gene was selected as the insertion site.

2.2.2.3 Construction of labeled plasmids

Using different strategies (see section **2.2.2.2**), recombinant plasmids were constructed by inserting marker genes to the infectious full-length cDNA clones of ACLSV. The plasmid of pDoc-G was the donor of EmGFP gene. The pK2GW7 was the donor of mCherry gene. The plasmid of p2488 was the donor of iLov gene (Table 2.2). The infectious full-length cDNA clones of ACLSV, i.e. pIF3-15 or pIF3-19, were used as backbones.

The plasmids were constructed using Gibson assembly or In-Fusion cloning (see section 2.2.1.6). The general working flow was similar to the construction of full-length cDNA clones of viruses (see section 2.2.2.1). First the target fragments having homologous ends of 15 bp were produced by PCR. In the PCRs, PRECISOR high-fidelity DNA polymerase was the enzyme of choice. The PCR reaction mixture and cycling conditions were set according to Table 2.16 and Table 2.17 (see section 2.2.1.3). Before cloning, the target fragments were gel purified (see section 2.2.1.5). The reaction mixture was directly used for transformation of competent *E. coli* cells by heat shock (see section 2.2.3.3). Positive colonies were selected by colony PCRs (section 2.2.1.3). In the PCRs, designed primers encompassed fused parts were used. The KAPA *Taq* DNA polymerase was used. The PCR reaction mixture and cycling conditions were set according to Table 2.14 and Table 2.15. The plasmids were isolated using QIAprep spin miniprep kit (Qiagen) according to the manufacturer's protocol. The insert parts were sequenced using Mix2Seq kit (see section 2.2.2.4). By comparison with the sequence of marker genes, the plasmids having correct sequence (no mutation in the insert) were agroinoculated to test-plants of *N. occidentalis* 37B (section 2.2.4.1).

2.2.2.3.1 Construction of plasmids using strategy A

To insert the marker gene of EmGFP to the 3' end of MP gene, two steps were conducted. As first step, EmGFP fragments with 15 bp extensions were amplified using primers #029 and #036. The plasmid pIF3-19 (Table 2.2) was linearized using primers #027 and #028. The linearized position was in front of the stop codon of MP gene, exactly between nucleotide (nt) 7090 and nt 7091 of genomic cDNA of ACLSV on pIF3-19 (see section 3.1.2). The two fragments were cloned using In-Fusion. The fusion reaction mixture consisted of 6 µl (215 ng) of linear pIF3-19, 2 µl (30 ng) of EmGFP with extensions and 2 µl of In-Fusion enzyme premix. Positive colonies were selected by colony PCR using primers #029 and #030. By sequencing the inserted parts, the plasmid having correct sequence of EmGFP was selected for next step and named pIF12-28. As second step, the plasmid pIF12-28 was linearized using

primers #027 and #037. The position was just behind the stop codon of the GFP gene in pIF12-28. The partial CP gene (plus the short fragment of 304 bp upstream) with extensions of 15 bp was amplified from pIF3-19 using primers #031 and #032. The target fragments were cloned using In-Fusion. The fuison mixture consisted of the linear pIF12-28 of 6.5 µl (350 ng), partial CP gene of 0.5 µl (60ng) and In-Fusion enzyme premix of 2 µl, H_2O filled up to 10 µl. Positive colonies were selected by colony PCR using primers #029 and #030. The fuison part was sequenced. Finally the plasmid having correct sequences was named pIF13-9.

To insert the marker gene of mCherry to the 5' end of CP gene, two steps were conducted. As first step, pIF3-19 was linearized using primers #064 and #065. The position was just in front of the start codon of the CP gene. The mCherry gene with extensions of 15 bp was amplified using primers #066 and #067. The fragments were cloned using Gibson assembly. The reaction mixture consisted of the linear pIF3-19 of 4.5 µl (126 ng), the mCherry with extensions of 1 µl (63 ng) and 5.5 µl Gibson master mix. The mixture was incubated for 2 h at 37°C, and used for transformation of competent *E. coli* NEB 5-alpha cells. The positive colonies were selected by colony PCR using primers #060 and #061. The plasmids of positive colonies were sequenced for the cloned part of the mCherry gene. The one with correct sequence was named pG5-4. As second step, the fragment of mCherry:CP (plus a short fragment of 304 bp upstream) containing extensions of 15 bp was amplified using primers #059 and #068. The plasmid of pG5-4 was used as templates. The fragment containing pV297:RdRp:MP: 3' untranslated region (UTR) was amplified using primers #054 and #071. The template was the linear pIF16-1 (section 2.2.2.3.3), which were produced by restriction digestion of plasmids pIF16-1 with *Xba*I. The target fragments were cloned using Gibson assembly. The reaction mixture consisted of the fragment of mCherry:CP of 1 µl (120 ng), the fragment containing pV297:RdRp:MP: 3' untranslated region of 9 µl (171 ng) and 10 µl Gibson master mix. The mixture was incubated for 2 h at 37°C, and used directly for transformation of *E. coli* Steller competent cells. Positive colonies were screened by colony PCR using primers #059 and #068. The fusion parts of the plasmids from positive colonies were sequenced. The expected plasmid was named pG11-15.

2.2.2.3.2 Construction of plasmids using strategy B

To prevent the deletion of inserts in ACLSV genomes, which could be caused by homologous recombination of the duplicated parts of overlapping ORFs, heterogeneous MP or CP genes were introduced into the genomic cDNAs of ACLSV.

Based on the plasmid of pIF13-9 (section 2.2.2.3.1), two other plasmids were constructed. In one case, the heterogeneous CP gene (plus a short fragment of 87 bp upstream) was amplified from pIF3-15 using primers #090 and #091. The plasmid of pIF3-15 was constructed during this thesis (see section 3.1.2). With the primers #090 and #091, an extension of 15 bp was added to the target CP genes. Using primers #087 and #088, the fragments of linear pIF13-9 excluding CP genes were generated. The target fragments were cloned using In-Fusion cloning. The reaction mixture consisted of the heterogeneous CP fragments of 0.4 µl (316 ng), the linear reduced pIF13-9 of 5.3 µl (250.2 ng) and In-Fusion enzyme premix of 1.4 µl. Positive colonies were selected using primers #099 and #110. The inserted part of the CP of pIF3-15 was sequenced for selecting clones without mutation(s). The expected plasmid was named pIF18-2. In the second case, partial fragment of pIF13-9 was amplified using primers #104 and #105. The fragments contained partial MP genes and the complete EmGFP genes. The 5' end of the partial MP genes started just behind the stop codon of RdRp genes. The plasmid of pIF3-15 was linearized using primers #106 and #107. The linear fragments contained all genes originally on pIF3-15 except for a partial MP gene. The target fragments were cloned using In-Fusion cloning. The reaction mixture consisted of 1 µl (326 ng) fragments of the partial MP gene plus EmGFP gene of pIF13-9, the linear fragments of partial pIF3-15 of 3 µl (393 ng) and the In-Fusion enzyme premix of 1 µl. Positive colonies were selected by colony PCR using primers #038 and #098. The cloning part of isolated plasmids was sequenced and the plasmid with correct sequence was named pIF25-7.

Based on the plasmid of pG11-15, two other plasmids were constructed. In the first case, the plasmid of pG11-15 was linearized using the primers #102 and #103. The linear pG11-15 contained all genes originally on it, except for the partial MP gene between the RdRp gene and the possible promoter (German et al. 1992) of the CP gene (304 bp upstream). Using primers #100 and #101, the partial MP gene on pIF3-15 was amplified with extensions of 15 bp at ends. The 5' end of partial MP gene was just behind the stop codon of the RdRp gene on pIF3-15. The 3' end of the partial MP gene was intact. The fragments were cloned using In-Fusion cloning. The reaction mixture consisted of the fragments of the partial pG11-15 of 5 µl (250 ng), the fragments of partial pIF3-15 of 1 µl (442 ng) and the In-Fusion premix of 1.5 µl. Positive colonies were selected using primers #096 and #097. The fused parts of the plasmids were sequenced. The expected plasmid was named pIF24-6. In the second case, the plasmid of pIF3-15 was linearized using primers #003 and #093. The linear fragments of pIF3-15 contained the original genes on it, except for the partial CP gene and 3' UTR. The partial CP gene was just behind the stop codon of MP gene. The fragments of mCherry:CP:3' UTR

genes were amplified from pG11-15 using primers #002 and #094. The target fragments were cloned using In-Fusion cloning. The reaction mixture consisted of 5 µl (370 ng) of the fragments of partial pIF3-15, 1 µl (426 ng) of the fragments of mCherry:CP:3' UTR and 1.5 µl of In-Fusion premix. Positive colonies were selected by colony PCR using primers #096 and #097. The cloning parts of isolated plasmids were sequenced. The expected plasmid was named pIF23-1.

2.2.2.3.3 Construction of plasmids using strategy C

The plasmid of pIF3-19 was linearized using primers #054 and #055. The position was just in front of the stop codon of CP gene of ACLSV. The mCherry with extensions of 15 bp was amplified using primers #056 and #057. The target fragments were purified and cloned using In-Fusion cloning. The reaction mixture consisted of the linear pIF3-19 of10 µl (50 ng), the mCherry of 1 µl (50 ng) and 2.2 µl of In-Fusion enzyme premix. Positive colonies were selected by colony PCR using primers #060 and #061. The fragment of the insert was sequenced and the plasmid with correct sequence was named pIF16-1.

Based on the plasmid of pIF16-1, the second plasmid was constructed. The plasmid of pIF16-1 was linearized using primers #061 and #055. The linearized position was the connection of CP and mCherry genes. Fragments of a linker of $(EAAAK)_4$ were produced by a PCR step (see section 2.2.1.3). The linear pIF16-1 and the linker were cloned using In-Fusion cloning. The reaction mixture consisted of 0.2 µl (53.6 ng) of the linker, 5.4 µl (310 ng) of the linear pIF16-1 and 1.4 µl of the In-Fusion enzyme premix. Positive colonies were selected by colony PCR using primers #080 and #110. The cloning part was sequenced, and plasmid with correct sequence was named pIF20-16.

Based on the plasmid of pIF16-1, the third plasmid was constructed. The plasmid of pIF16-1 was linearized using the primers #083 and #084. The linear fragments contained all the original genes on pIF16-1, except for the gene of mCherry. The fragments of iLov gene were amplified using primers #085 and #086. Extensions of 15 bp were added to the ends of iLov genes. The target fragments were cloned using In-Fusion cloning. The reaction mixture consisted of 1 µl (254 ng) of the iLov fragments, 6 µl (258 ng) of the linear pIF16-1 and 1.8 µl of the In-Fusion premix. The positive colonies were selected using primers #085 and #086 (annealing at 61°C). The cloning part of the plasmids was sequenced for selecting clones without mutation(s). The expected one was named pIF27-10.

2.2.2.4 Sequencing of obtained plasmids

For the purpose of sequencing, the plasmids in a volume of 15 µl were mixed with sequencing primers (see Supplementary Table S1) of 2 µl (10 µM) in a Mix2Seq tube (see information about the Mix2Seq kit in Table 2.10). The samples were then sent to Eurofins Genomics for sequencing. The first sequencing primers of #111 and #112 were bound to vector pV297. The next primers were designed step by step based on the obtained sequence data. The obtained partial fragments were assembled using SeqMan (DNASTAR Inc.) based on the overlapping data. The sequences of the complete genomic cDNAs were then annotated using the NCBI Basic Local Alignment Search Tool (BLAST) and NCBI Conserved Domain Search. The phylogeny analyses were performed between the obtained sequences and the published sequences in NCBI database using MEGA5 (Table 2.11).

2.2.3 Microbiology

2.2.3.1 Preparation of electrocompetent bacteria cells

Electrocompetent cells of *A. tumefaciens* GV2260, *A. tumefaciens* ATHV and *E. coli* NM522 were used in the present work. For preparation of electrocompetent *A. tumefaciens* GV2260, the cells were plated on a yeast extract broth (YEB) agar plate containing 25 µg/ml rifampicin and 50 µg/ml carbenicillin and grown at 28°C for around 45 hours. And then 50 ml YEB medium containing the same antibiotics were inoculated with one single colony picked from the plate and again grown at 28°C with 200 rpm for around 45 hours. Then 6 flasks with 250 ml YEB medium containing the same antibiotics were inoculated with 5 ml of the pre-culture and incubated at 28°C until the OD_{600} value reached 0.5. The culture was incubated on ice for 15 min and then harvested by centrifugation for 15 min at 4,000 rpm and 4°C. The bacterial pellets were resuspended in 250 ml ice-cold sterile Mili-Q H_2O. This wash step was repeated once. After that the pellet was resuspended in 200 ml ice-cold sterile 10% glycerol. The resuspending was centrifuged again. By discarding the supernatant, the volume of the wet pellet was reduced to 1 to 3 ml. The pellet was mixed with the liquid by gently hand-vortex. Aliquots of 50 µl bacteria were frozen in liquid nitrogen and immediately transferred to -80°C until electroporation experiments.

The electrocompetent *A. tumefaciens* ATHV and *E. coli* NM522 cells were equally prepared. Cultivation of *A. tumefaciencs* ATHV was performed on YEB medium with 25 µg/ml rifampicin at 28°C. *E. coli* NM522 was cultivated on Luria-Bertani (LB) medium at 37°C without antibiotics.

2.2.3.2 Electroporation

For transformation of electrocompetent *Agrobacterium* cells, an aliquot of 50 µl electrocompetent cells was thawed on ice. Then plasmids of 2 µl were slightly mixed with the cells. Immediately the mixture was transferred to a pre-cooled electroporation cuvette (2 mm gap). The cuvette was placed into a Gene pulser electroporation system and pulsed with 2500 V, 25 µF and 200 Ω. Once done 950 µl of prewarmed SOC (super optimal broth with catabolite repression) medium was added to the bacteria. The culture was transferred to a 2 ml reaction tube and was incubated for 2 hours at 28°C and 200 rpm. Afterwards, culture of 10 µl and 50 µl was plated on antibiotic-containing YEB agar plates, respectively. The remaining culture was centrifuged for 30 sec at 4000 rpm. The pellet was resuspended in 100 µl medium and also plated. Plates were incubated at 28°C for above 40 hours. Single colonies were picked for further experiments.

Transformation of electrocompetent *E. coli* NM522 cells was performed in the same way. The optimal incubation temperature was 37°C and LB medium was used. The cells were agitated for 1 hour at 37°C and 200 rpm after electroporation. The culture of 50 µl, 100 µl and rest were plated on LB agar plates, respectively. The plates were incubated overnight at 37°C.

2.2.3.3 Heat shock

In the present work, commercial chemo-competent *E. coli* cells were used, including NEB 5-alpha, NEB 10-beta and Steller (Table 2.1). Unless mentioned otherwise NEB 10-beta was the cells of choice. The transformation was performed mainly according to the manufacturer's protocols. The competent cells were thawed on ice. Five µl of a Gibson or In-Fusion reaction mixture was added into 50 µl of the thawed cells. The mixture was incubated for 30 min on ice. Then a heat shock was performed for 30 to 45 sec in a water bath at 42°C, and the cells were then cooled down on ice for 2 to 5 min. Afterwards the cells were incubated in 950 µl prewarmed SOC medium for 1 hour at 37°C and 200 rpm. The culture of 100 µl, 200 µl and rest was plated on LB agar plates containing 30 µg/ml kanamycin, respectively (see section 2.2.3.2). The plates were incubated overnight at 37°C. Single colonies were picked for further experiments.

2.2.4 Inoculation of plants

2.2.4.1 Mechanical agroinoculation

To test the infectivity of the constructed full-length cDNA clones of viruses, they were agroinoculated to test-plants. The herbaceous test-plants included *Nicotiana occidentalis* 37B and *Chenopodium quinoa* (section 2.1.2).

To prepare the inocula of agrobacteria containing the tested cDNA clones, a single colony (section 2.2.3.2) was inoculated to 50 ml antibiotic-containing YEB medium (Table 2.8). The culture was incubated for around 45 hours at 28°C and 190 rpm. The agrobacteria were harvested by centrifugation for 15 min at 5000 rpm, and then the pellet was resuspended in 10 mM $MgSO_4$. This suspension was inoculated directly to test-plants.

To perform a mechanical inoculation, the upper surface of leaves was dusted with carborundum (600 mesh). After that the inoculum was rubbed onto the leaf surface. On each leaf, about 500 µl of inoculum was used. Symptom development was observed during three weeks.

2.2.4.2 Sap inoculation

To consequently transmit the viruses generated from infectious full-length cDNA clones to new host plants, sap inoculation was conducted. The juice of infected leaves was extracted as inoculum. To prepare this, fresh symptomatic leaf blades were ground in herb-herb inoculation buffer (Table 2.8) in a universal extraction bag. Approximately 1 g fresh leaf blades was ground in 100 ml inoculation buffer, the amount of leaf materials and buffer can be increased in proportion. The juice (about 200 µl) was then slightly rubbed onto leaf surfaces of test-plants that were dusted with carborundum (600 mesh). Symptom development was observed during three weeks.

2.2.4.3 Agroinoculation by vacuum infiltration

To inoculate woody plants, the protocol of agroinoculation of infectious cDNA clones by vacuum infiltration was developed in the present work.

The seedlings of one to three-month old *Malus domestica* cv. Golden Delicious were used as test-plants (section 2.1.2). Before suffering vacuum infiltration, the brown seed coats on cotyledons were removed, and the seedlings were taken to room temperature (ca. 20°C) for around 20 hours under natural light. Wounded or unwounded seedlings were used in the

experiments. Of wounded seedlings, each cotyledon was stuck by a sterilized needle to introduce four to six pin holes (Figure 2.2A and B). The entire wounded or unwounded seedlings were immersed in sterilized Milli-Q water containing 1% (v/v) Tween 80 for 10 min, and rinsed two times with sterilized Milli-Q water. Prepared seedlings were then immediately immersed in the inoculum for vacuum infiltration.

The inocula of transformed *A. tumefaciens* containing test cDNA clones were prepared as described above (sections 2.2.3.2 and 2.2.4.1), additionally 0.2% (v/v) Tween 20 was added.

The vacuum was generated in a closed desiccator using a Büchi V-500 vacuum pump with vacuum controller B-721 (Table 2.9) (Figure 2.2C and D). Different pressure and duration of vacuum were used in independent treatments.

The infiltrated seedlings were immediately planted in virus-free soil after the vacuum infiltration and were grown in an insect-proof glasshouse at 60-65% humidity and 20-25°C.

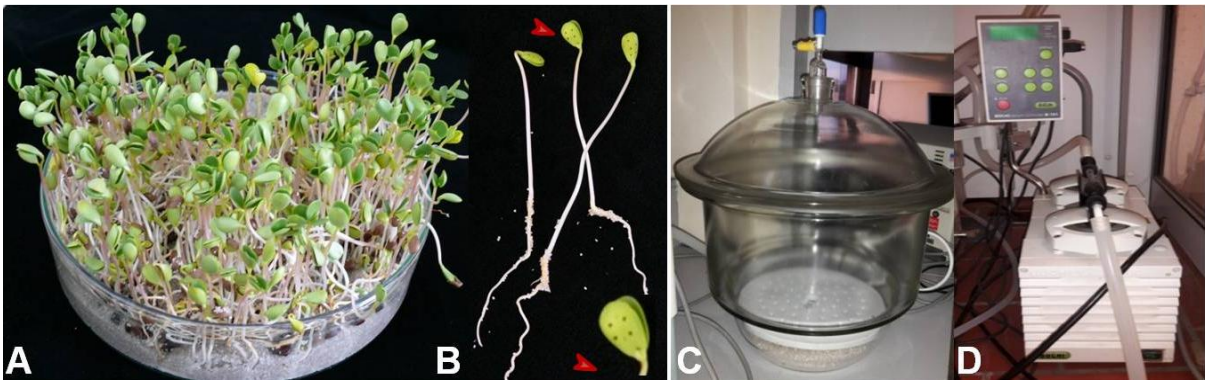

Figure 2.2 Apple seedlings and pump system used in vacuum infiltration. Apple seedlings with unwounded (A) and wounded (B) cotyledons were used in the vacuum infiltration. Red arrow indicates a magnification of a wounded cotyledon. The vacuum system consisted of a dessicator (C) and a pump system (D).

2.2.4.4 Grafting

To transmit virus particles from agroinfected apple to healthy apple, cleft grafting was conducted. The rootstocks (ca. 25 cm) were prepared from one-year old apple seedlings (ca. 70 cm) (see section 2.1.2). The top branches of the seedlings were removed using sterilized guarder knife and the rootstocks were split 3 to 5 cm. The scions were prepared from the agroinfected apple seedlings (see section 2.2.4.3). The selected branches of ca. 8 cm were pruned off the apple seedlings, and a V-shaped appearance that will fit tightly into the split on the stocks were made by long tapering cuts on both sides of the bottom of the sticks. The scions were then carefully placed into the split of rootstocks, and the graft unions were wrapped with parafilm and rubber. The top of the scions was sealed with grafting wax

(Maywax). The grafted plants were kept in an insect-proof glasshouse with 60-65% humidity at ca. 22°C.

2.2.4.5 Detection of viral infection in plants

The host-plants were tested by RT-PCR for virus infection. First total RNAs of noninoculated leaves were extracted using silica capture method (section 2.2.1.1). And then the cDNAs templates were produced from total RNAs using ReverseAid RTase (section 2.2.1.2). Virus fragments were detected using selected primers: for ACLSV detection, the primers #046/#047 were used; for detection of ASPV, the primers #048/#006 were used. PCR reaction mixture and cycling conditions were described in section 2.2.1.3.

2.2.5 Protein immunoblot

2.2.5.1 Protein preparation

Fresh leaf blades were ground in 1× protein extraction buffer (Table 2.8) using a mortar and a pestle (250 mg leaf blades : 250 µl buffer). The ground plant materials were centrifuged for 3 min at 13000 rpm. The supernatant of 200 µl was transferred into a new reaction tube. The supernatant was centrifuged for 3 min at 13000 rpm, and then 150 µl were transferred into another reaction tube.

The raw protein extraction of 40 µl was transferred into a reaction tube containing 1× protein loading buffer (Table 2.8). The mixture was incubated for 10 min at 99°C.

2.2.5.2 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

To separate the proteins (section 2.2.5.1) extracted from test-plants, SDS-polyacrylamide gels (PAGs) were prepared. Firstly resolving gels of 12% SDS-PAGs were prepared in SE245 dual gel casters. Four volumes of resolving gels consisted of 8.7 ml of H_2O, 5 ml of 1.5 M Tris (pH 8.8, plus 1% SDS), 6 ml of Rotiphorese® Gel 40 (37.5:1), 20 µl of tetramethylethylenediamine (TEMED) and 100 µl of 10% ammonium persulfate (APS). The gel pre-mixture was poured between the two glass plates and overlaid with isopropanol. Once the resolving gel was polymerized, the isopropanol was removed. The stacking gel was poured and a comb with the required number of wells (10 or 15) was inserted. Four volumes of stacking gels consisted of 6.43 ml of H_2O, 2.5 ml of 0.5 M Tris (pH 6.8, plus 1% SDS), 1

ml of 40% Rotiphorese® Gel 40 (37.5:1), 10 µl of TEMED, 50 µl of 10% APS and 50 µl blue loading dye.

Electrophoresis was performed using a SE250 vertical electrophoresis system with a Consort E844 power supply (Table 2.9). The gels ran in SDS-PAGE running buffer (Table 2.8) for 1.5 hours at 150 V. After the run the gels were processed for western blotting (see section 2.2.5.4).

2.2.5.3 Transmembrane

After SDS-PAGE electrophoresis (see section 2.2.5.2), the separated proteins were transferred to an Immobilon-P transfer membrane through tank blotting. The transfer was conducted using a mini Trans-Blot transfer cell with a Consort E844 power supply. The resolving gels were cut off from the glass plates. Each gel was assembled with the membrane, filter papers and foam pads in a tank transfer cassette holder. Before the assembly the filter papers and foam pads were equilibrated in transfer buffer. The blotting was performed in iceblock-cooling 1× transfer buffer for 1.5 hours at 120 V. The efficiency of transfer was determined through PageRuler prestained protein ladder (Table 2.4).

2.2.5.4 Western blot

The membranes were blocked in 1x PBS-T buffer containing 5 % (w/v) milk powder for around 1 hour at room temperature with shaking. The membranes were then washed 3 x 15 min with 1x PBS-T buffer. Afterwards the washed membranes were incubated with diluted primary antibodies overnight at 4°C. The primary antibodies were diluted in blocking buffer according to Table 2.6. Next day, the membranes were washed 3x 15 min with AP buffer. During the wash step, secondary antibodies were diluted in blocking buffer according to Table 2.6. The membranes were incubated in secondary antibody dilution for at least 1 hour at room temperature with shaking. After a subsequent washing step, the membranes were visualized. The membranes were washed 3 x 10 min in AP buffer. To visualize, the membrane was incubated for 2 min with BCIP/NBT reagent.

2.2.6 Immunosorbent electron microscopy (ISEM)

Viral particles of ACLSV were visualized from *N. occidentalis* 37B and G. Delicious test-plants by ISEM as described previously (Jelkmann et al. 1990). The polyclonal antiserum used was ACLSV-AS1236 (JKI, Institute for Epidemiology and Pathogen Diagnostics). For ISEM the antiserum was diluted 1:1000 in 0.1 M K-Na phosphate buffer pH 7.0. Nickel grids

were incubated for 5 min at room temperature in the diluted antiserum, and then were washed with 1.5 ml the same buffer. The grids were transferred to leaf homogenate and incubated overnight at room temperature. Decoration of particles was performed by incubation in 1 : 50 diluted antiserum at room temperature for 15 min. Excess liquid was carefully removed with filter paper, and the grids were washed with 7% uranyl acetate in ultrapure water. Dry grids were examined with a Zeiss EM 10C electron microscope.

3. Results

3.1 Construction of full-length cDNA clones

In total 17 full-length cDNA clones were obtained in the present work. In each plasmid the viral genomic cDNA was inserted between the CaMV 35S promoter and HDVpA (Figure 3.1). For each virus isolate, the number of obtained clones is shown in Table 3.1.

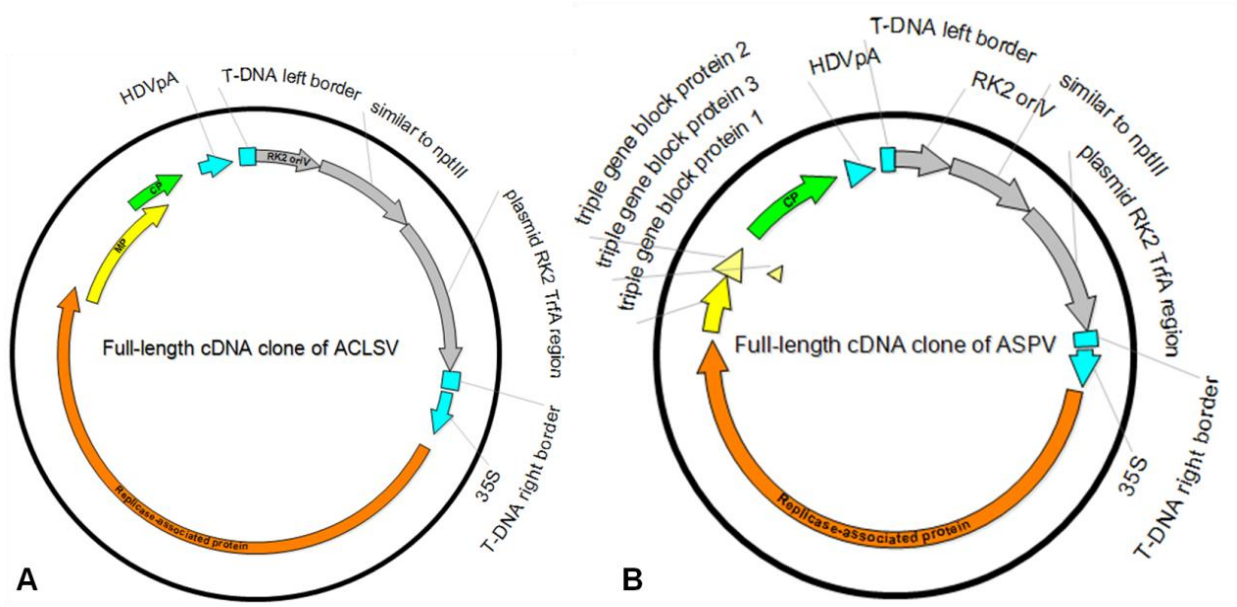

Figure 3.1 The structures of the constructed full-length cDNA clones. The genomic cDNAs of ACLSV (A) and ASPV (B) are inserted between a CaMV 35S promoter and an HDVpA.

Table 3.1 Number of full-length cDNA clones constructed using different methods

Virus species	Isolates	Number of obtained clones		
		CPEC	Gibson	In-Fusion
ACLSV	(27)/85	1	1	3
ACLSV	38/85	4	-	4
ACLSV	(36)/88	-	-	2
ASPV	40/87	-	-	2

3.1.1 Construction of ACLSV clones isolate (27)/85

In total five clones were obtained for ACLSV isolate (27)/85 (Table 3.1). One was obtained using CPEC method, one was obtained using Gibson assembly and three were obtained using In-Fusion cloning. The preparation of the genomic cDNAs and linear pV297 were as described in section 2.2.2.1.

In CPEC, 5 µl (43 ng) of genomic cDNA fragments and 3 µl (42 ng) of linear pV297 were used in the reaction mixture (see section 2.2.1.6). After transformation of NEB 5-alpha *E. coli* cells, 50 colonies developed on LB plate inoculated with 100 µl transformants. Using primers #009/#010, #011/#012, and #046/#047, respectively (section 2.2.1.3), PCR products of about 600, 340 and 680 bp were amplified from 1 out of 25 colonies. The plasmid was named pCPEC2-1.

In Gibson assembly, the reaction mixture consisted of 3.5 µl (200 ng) of genomic cDNA fragments, 0.8 µl (100 ng) of linear pV297 and 10 µl 2× Gibson MasterMix, H_2O filled up to 20 µl. The reaction mixture was incubated for 1 hour at 50°C. The mixture of 5 µl was directly used for transformation of NEB 10-beta *E. coli* cells. By colony PCRs with primer pairs of #009/#010, #011/#012 and #046/#047 (section 2.2.1.3), 1 positive colony was selected out of 27 colonies. The plasmid was named pG2-138.

In In-Fusion cloning, the reaction mixture consisted of 8 µl (100 ng) of genomic cDNA fragments, 3 µl (51 ng) of linear pV297 and 2.75 µl of 5× In-Fusion enzyme premix. The reaction mixture was incubated for 1 hour at 50°C. The mixture of 5 µl was directly used for transformation of NEB 10-beta *E. coli* cells. Three positive colonies were selected out of 50 colonies by colony PCR using primers primer pairs of #009/#010, #011/#012 and #046/#047 (section 2.2.1.3). The plasmids were named pIF15-13, pIF15-15 and pIF15-26.

3.1.2 Construction of ACLSV clones isolate 38/85

Eight clones were obtained for ACLSV isolate 38/85 (Table 3.1). Four clones were obtained using CPEC method, and the others were obtained using In-Fusion cloning. The preparation of genomic cDNAs and linear pV297 was as described in section 2.2.2.1.

Two independent CPEC were performed. In the two CPEC assays, the reaction mixture was prepared according to Table 2.18, except for the amount of inserts and vectors. In the first CPEC, 10 µl (55 ng) of genomic cDNA fragments and 0.5 µl (135 ng/ µl) of linear pV297 were used in the reaction mixture. In the second CPEC, 10 µl (55 ng) of genomic cDNA

fragments and 3.6 µl (18.7 ng/µl) of linear pV297 were used in the reaction mixture. The cycling conditions were shown in Table 2.19. The reaction mixture of 5 µl was used for transformation of NEB 10-beta *E. coli* cells. The colony PCR assays were performed using primer pairs of #009/#010, #011/#012 and #046/#047 according to section 2.2.1.3. Finally, two positive colonies were selected out of more than 300 colonies in the first CPEC. Two other positive colonies were selected out of around 100 colonies in the second CPEC. The plasmids were named pCPEC6-11, pCPEC6-18, pCPEC7-39 and pCPEC7-91.

In In-Fusion cloning, the reaction mixture consisted of 10 µl (55 ng) of genomic cDNA fragments, 3.6 µl (67 ng) of linear pV297 and 3.4 µl of 5× In-Fusion enzyme premix. After incubation for 2 hours at 50°C, the reaction mixture of 3.5 µl was directly used for transformation of NEB 10-beta *E. coli* cells. Finally, 4 positive colonies were selected out of the 36 colonies by colony PCR using primer pairs #009/#010, #011/#012 and #046/#047 (see section 2.2.1.3). The plasmids were named pIF3-12, pIF3-14, pIF3-15 and pIF3-19.

3.1.3 Construction of ACLSV clones isolate (36)/88

Two clones of ACLSV isolate (36)/88 were constructed using In-Fusion cloning (Table 3.1). The preparation of genomic cDNAs and linear pV297 were described in section 2.2.2.1. The reaction mixture consisted of 3 µl (51 ng) of genomic cDNA fragments, 7 µl (101 ng) of linear pV297 and 2.5 µl of 5× In-Fusion enzyme premix. The reaction mixture was incubated for 1 hour at 50°C. Afterwards the reaction mixture of 5 µl was directly used for transformation of NEB 10-beta *E. coli* cells. Two positive colonies were selected out of 90 colonies by colony PCR using primers #009/#010, #011/#012 and #046/#047 (see section 2.2.1.3). The plasmids were named pIF14-14 and pIF14-23.

3.1.4 Construction of ASPV clones isolate 40/87

Two clones of ASPV isolate 40/87 were constructed using In-Fusion cloning (Table 3.1). The preparation of genomic cDNAs and linear pV297 were as described in section 2.2.2.1. The reaction mixture consisted of 15 µl (90 ng) of genomic cDNA fragments, 3 µl (54 ng) of linear pV297 and 4.5 µl of 5× In-Fusion enzyme premix. The reaction mixture was then incubated for 2 hours at 50°C. After that the mixture of 4 µl was used for transformation of NEB 10-beta *E. coli* cells. By colony PCR using primers #013/#014, #015/#016 and #048/#049 (see section 2.2.1.3), fragments of around 480, 500 and 360 bp were amplified from two out of 23 colonies. The plasmids were named pIF4-4 and pIF4-16.

3.2 Infectivity of the constructed clones on herbaceous plants

To test the infectivity, the obtained full-length cDNA clones of ACLSV and ASPV were agroinoculated to ten plants of *Nicotiana occidentalis* 37B and ten plants of *Chenopodium quinoa*, respectively. The inoculum of pV297-transformed agrobacteria was used as a negative control. The agroinoculation was performed according to section 2.2.4.1. The tests were performed independently three times.

After agroinoculation the symptom development was observed every day during three weeks. The infection of viruses was determined by RT-PCR (section 2.2.4.5). In PCR positive plants the existence of virus particles was confirmed by ISEM (section 2.2.6.1).

The results of agroinoculation demonstrated that four full-length cDNA clones were infectious on *N. occidentalis* 37B, i.e. pIF3-15, pIF3-19, pIF4-4 and pIF14-23. But no infection was observed for any clone on the test-plants of *C. quinoa* after the agroinoculation.

Moreover, infected *N. occidentalis* 37B were further inoculated to healthy *N. occidentalis* 37B plants by sap inoculation with 100% successful rate. No transmission was observed on *C. quinoa* by sap inoculation.

3.2.1 Symptoms development on *N. occidentalis* 37B

On *N. occidentalis* 37B test-plants, symptoms caused by pIF3-15, pIF3-19 and pIF14-23 were similar. For each clone, all test-plants were infected via agroinoculation. Seven to nine days after inoculation, infected plants developed the first symptom of yellow spots on noninoculated leaves. Over time symptoms of chlorosis, crinkle and slight necrotic spots were observed on upper leaves (Figure 3.2). No symptom developed on the inoculated leaves.

The clone of pIF4-4 caused systemic symptoms on all the ten test-plants of *N. occidentalis* 37B after mechanical agroinoculation. Eight to nine days after inoculation, symptoms started to appear on the noninoculated leaves. The symptoms included chlorosis and vein-banding on upper leaves (Figure 3.3). No symptoms developed on the inoculated leaves.

The control plasmid pV297 did not cause symptoms on test-plants. The following inoculations remained without symptoms on test-palnts: pCPEC2-1, pG2-138, pIF15-13, pIF15-15, pIF15-26, pCPEC6-11, pCPEC6-18, pCPEC7-39, pCPEC7-91, pIF3-12, pIF3-14, pIF14-14 and pIF4-16. They also tested negative in PCR.

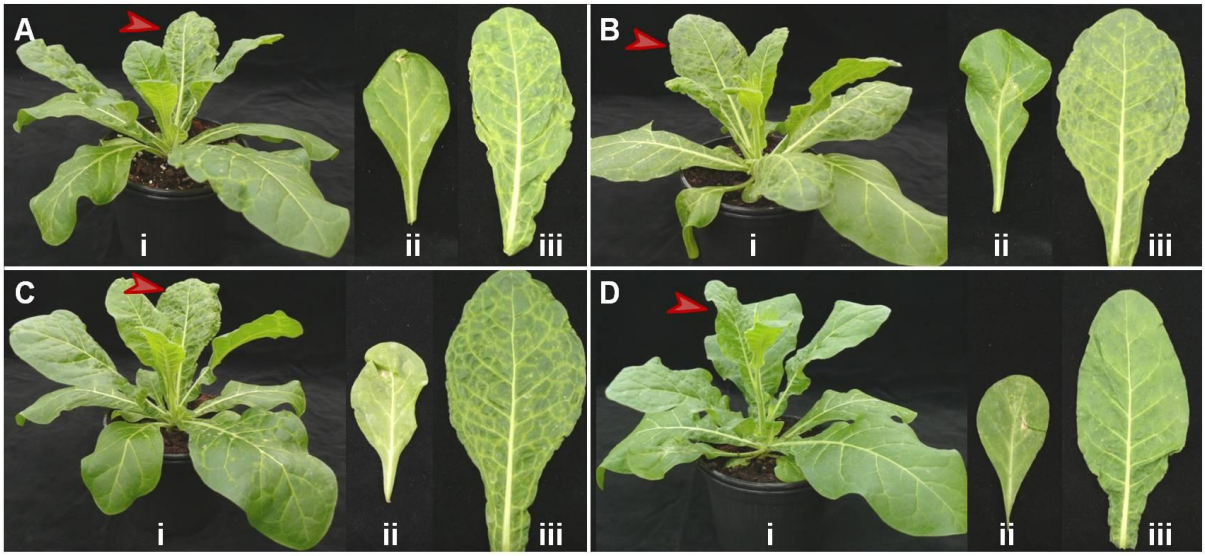

Figure 3.2 Symptoms on *N. occidentalis* 37B 18 days after agroinoculation with ACLSV clones. The plants were agroinoculated with the infectious clones of pIF3-15 (A), pIF3-19 (B), pIF14-23 (C) and pV297 (D) respectively. Symptoms of mosaic and chlorosis were observed on plants inoculated with pIF3-15 (A), pIF3-19 (B), pIF14-23 (C). No infection was observed on the plants inoculated with pV297 control (D). i was the side view of the whole infected plants. ii was one of the symptomless inoculated leaves of each plant. iii was a separated leaf indicated by red arrow on symptomatic plants.

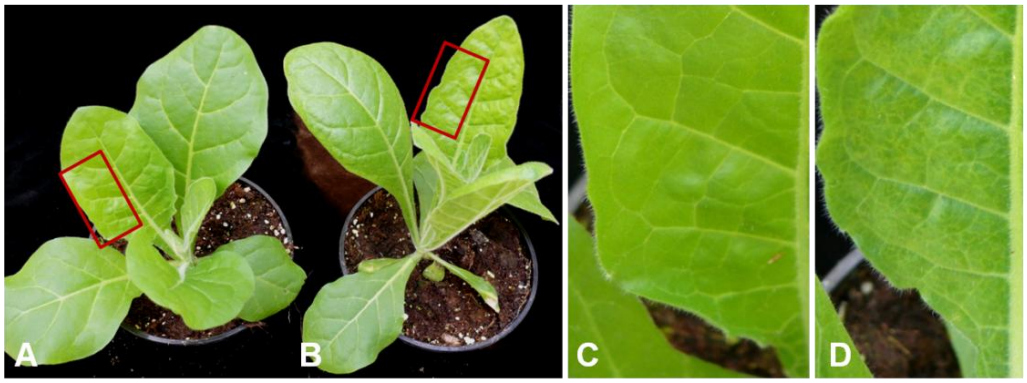

Figure 3.3 Symptoms on *N. occidentalis* 37B 10 days after agroinoculation with the ASPV clone. The plants were agroinoculated with control pV297 (A) and pIF4-4 (B) respectively. No symptoms developed on the pV297-inoculated plants (A and C). The symptoms of chlorosis and vein-banding were observed on pIF4-4 inoculated plants (B and D). C and D are magnifications of the red box- indicated in A and B respectively.

3.2.2 RT-PCR detection of viruses in agroinoculated test-plants

The agroinoculated plants were analyzed by RT-PCR for ACLSV and ASPV infections (section 2.2.4.5). In symptomatic plants agroinoculated with pIF3-15, pIF3-19 and pIF14-23, PCR fragments of around 650 bp were amplified using primers #046/#047. The results suggested the infection of ACLSV in the test-plants. In the plants inoculated with control pV297, no PCR product was generated using the same primers. All inoculated test-plants with the other 12 plasmids of the full-length clones of pCPEC2-1, pG2-138, pIF15-13, pIF15-15,

pIF15-26, pCPEC6-11, pCPEC6-18, pCPEC7-39, pCPEC7-91, pIF3-12, pIF3-14 and pIF14-14 remained negative in PCR. This result indicated that no ACLSV infection was established from the agroinoculation of the 12 clones in the test-plants.

In the symptomatic plants agroinoculated with pIF4-4, the band of around 430 bp was amplified using primers #048/#006, suggesting the existence of ASPV in the inoculated plants. In the plants inoculated with pV297, no PCR product was generated using primer pair #048/#006. In the test-plants inoculated with pIF4-16, no PCR product of around 430 bp was generated. This result indicated no ASPV infection was caused by pIF4-16 in the test-plants.

No infection of ACLSV or ASPV was detected in the test-plants of *C. quinoa* by RT-PCR. This result suggested that no infection of ACLSV or ASPV was established in the *C. quinoa*.

3.2.3 Detection of viral particles by ISEM

Virus particles of ACLSV in the symptomatic and PCR-positive *N. occidentalis* 37B plants were detected using ISEM (section 2.2.6.1). The virions were very flexuous filaments, the length of the virions ranged from 840 nm to 1210 nm. The width was approximately 12 nm (Figure 3.4).

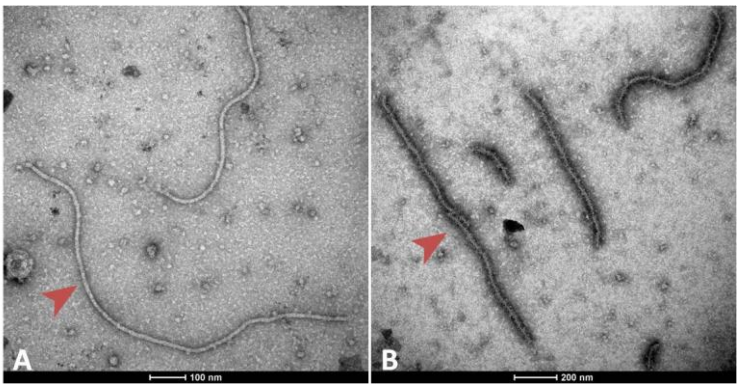

Figure 3.4 Virions of ACLSV detected in infected *N. occidentalis* 37B using ISEM. A: virions trapping with antiserum was used as standard, scale bar = 100 nm. B: virions definitively decorated with antiserum; scale bar = 200 nm. Red arrows indicated virions.

3.2.4 Transmission of viruses by sap inoculation

ACLSV generated from infectious clones of pIF3-15, pIF3-19 and pIF14-23 in infected *N. occidentalis* 37B plants can be further transmitted to healthy *N. occidentalis* 37B plants. The efficiency of transmission was 100%. The symptoms on the sap-inoculated plants were more severe than those on the agroinoculated plants.

ACLSV extracts from *N. occidentalis* 37B were rubbed onto *C. quinoa* plants as well. However, the establishment of infection was inconclusive. Among three trials, one time the infection was successful in test-plants. In the successful one, a positive PCR result was obtained in two out of four plants for pIF3-15 and pIF3-19, respectively, as well as in one out of four plants for pIF14-23. Systemic symptoms of chlorosis and necrotic spots were observed on the infected plants (Figure S1).

Similar to the ACLSVs, the ASPV generated from pIF4-4 can be further mechanically transmitted to *N. occidentalis* 37B by sap inoculation at 100% infection rate. No transmission to *C. quinoa* was successful.

3.3 Agroinoculation of woody plants with infectious clones

To inoculate woody hosts, the infectious full-length cDNA clones of pIF3-15, pIF3-19, pIF4-4 and pIF14-23 were first mechanically agroinoculated upon the one-year-old G. Delicious, *P. persica* and *P. armeniaca* respectively. At least eight plants were agroinoculated in each treatment. However, two months after the inoculation, no viral infection was detected in the test-plants by RT-PCR (section 2.2.4.5).

The infectious full-length cDNA clones of pIF3-15, pIF3-19, pIF4-4 and pIF14-23 were then agroinoculated to three-month-old apple seedlings of G. Delicious by vacuum infiltration. Except for the parameters of air pressure and duration of vacuum time, the protocol of vacuum infiltration was described in section 2.2.4.3.

Different parameters were used in three vacuum infiltration assays. In the vacuum infiltration 1, wounded and unwounded apple seedlings were used as the host plants. The infectious full-length cDNA clone of pIF3-19 was used as inoculum, whilst pV297 was used as a negative control. The vacuum infiltration was conducted for 10 min at 50 hPa. In the vacuum infiltration 2, the wounded apple seedlings were used as host plants. The inocula contained cDNA clones pIF3-15, pIF3-19 and pV297, respectively. The infiltration was performed at 500 hPa for 2, 5 and 10 min, respectively. In the vacuum infiltration 3, the wounded apple seedlings were used as host plants. The inoculums contained pIF3-15, pIF3-19, pIF14-23, pIF4-4 and pV297, respectively. The infiltration was performed for 5 min at 500 hPa.

The infection of viruses in infiltrated apple seedlings was detected two months after inoculation by RT-PCR (section 2.2.4.5). In PCR-positive samples, the existence of viral particles was confirmed by ISEM (section 2.2.6.1).

3.3.1 Infectivity of the clones on apple seedlings

In vacuum infiltration 1, 23 wounded and 35 unwounded apple seedlings were agroinoculated with the infectious clone of pIF3-19. Two weeks after the infiltration, most of the 35 grew well, whilst nearly half of the 23 wounded seedlings wilted and died. Around 50 days after the infiltration, 5 out of the 23 and 18 out of the 35 seedlings survived (Figure 3.5A). ACLSV infection in survived seedlings was then detected by RT-PCR. The PCR results indicated that 4 out of the 5 seedlings and 2 out of the 18 seedlings were positive for ACLSV infection (Figure 3.5A). Three of the four PCR-positive seedlings were analyzed by ISEM for viral particles, and ACLSV particles were observed in the samples (data not shown). One PCR negative sample was tested as well and no viral particle was observed (data not shown).

In the control group, ten wounded and nine unwounded apple seedlings were agroinoculated with pV297. Two weeks after the infiltration, all seedlings died.

In vacuum infiltration 2, 15 or 10 wounded seedlings were agroinoculated with the infectious clones of pIF3-15, pIF3-19 and control pV297, respectively. The duration of vacuum time was 2, 5 and 10 min, respectively. After the infiltration most of the seedlings grew well, only a few wilted in two weeks. Around 50 days after the infiltration the survival rates, defined as the ratio of the number of survived seedlings to the number of infiltrated seedlings, of above 66% (10/15, pIF3-19) were observed for all the tests (Figure 3.5B). The PCR-positive rates, defined as the ratio of the number of the PCR-positive samples to the number of the survived seedlings, of above 21% (3/14, pIF3-15) were observed (Figure 3.5B) for all the tests.

In vacuum infiltration 3, 10, 15 or 20 wounded seedlings were agroinoculated with the infectious clones of pIF3-15, pIF3-19, pIF14-23, pIF4-4 and control pV297, respectively. The results are shown in Figure 3.5C. After the infiltration most seedlings grew well, showing survival rates of above 70% (7/10, pV297). For pIF3-15, the PCR-positive rate was 78% (11/14). For pIF3-19, the PCR-positive rate was 87% (7/8). For pIF4-4, the PCR-positive rate was 50% (9/18). For pIF14-23, the PCR-positive, however, was 25% (2/8). For pIF3-15 and pIF3-19, three out of the PCR-positive seedlings were detected by ISEM: ACLSV particles were observed in the samples (data not shown). ASPV particles could not be observed in the

PCR-positive samples of pIF4-4 inoculated. One PCR negative sample was analyzed as well and no viral particle was observed.

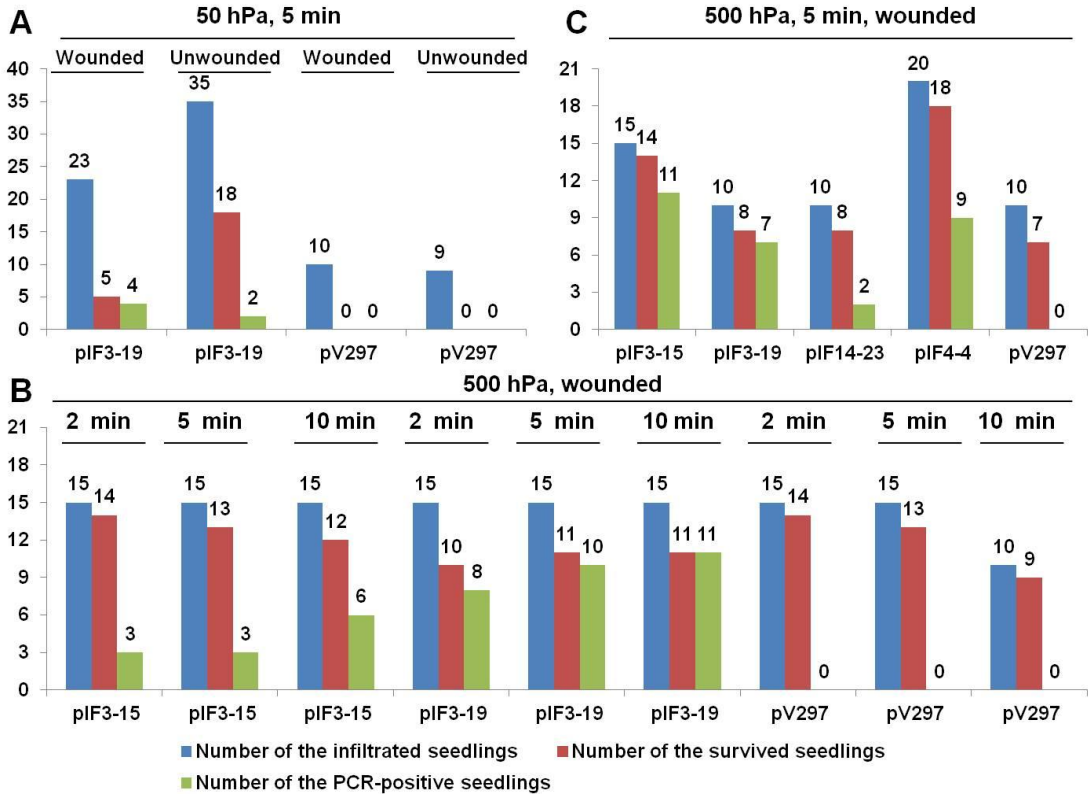

Figure 3.5 The efficiency of agroinoculation of apple seedlings with obtained infectious cDNA clones by vacuum infiltration. A: inoculation of wounded and unwounded seedlings with infectious clone pIF3-19, the vacuum infiltration was performed at 50 hPa for 5 min. B: inoculation of wounded apple seedlings with infectious clones pIF3-15 and pIF3-19, respectively; the infiltration was performed at 500 hPa for 2, 5 and 10 min respectively. C: vacuum infiltration of wounded apple seedlings at 500 hPa for 5 min with pIF3-15, pIF3-19, pIF14-23 and pIF4-4, respectively. The vector pV297 was used as a negative control. The number of survived seedlings and the number of PCR-positive seedlings were obtained around 50 days after the vacuum infiltration.

3.3.2 Transmission of viruses to woody plants by grafting

To test the transmission of the viruses generated from the infectious full-length cDNA clones of pIF3-15, pIF3-19 and pIF4-4 in apple seedlings (section 3.3.1), grafting was performed (section 2.2.4.4).

To test the transmission of ACLSVs, the branches of virus-infected apple seedlings were used as scions for grafting. Healthy one-year-old apple seedlings were used as rootstocks. Six grafted plants were produced for each ACLSV isolate (clone pIF3-15 and pIF3-19). Four plants were propagated using the infected scions and two as healthy controls. Four months after the grafting, the grafted plants were analyzed for ACLSV infection by RT-PCR (section 2.2.4.5). The PCR results demonstrated that all four trees grafted by virus-infected scions

were successfully infected with the viruses. In addition one of the PCR-positive samples was analyzed by ISEM. The existence of ACLSV particles was confirmed in the test-sample.

Transmission of ASPV was tested in the same way as just described for ACLSV. Nine grafted plants were produced, containing eight grafts using the infected scions and one using the healthy scion. Three months after grafting, ASPV infection in the grafted plants was detected by RT-PCR (section 2.2.4.5). The eight trees grafted with the infected scions were PCR-positive. But in the ISEM, no viral particles were observed in the PCR-positive samples.

3.4 Sequence analysis

The genomic cDNAs of the infectious full-length cDNA clones of pIF3-15, pIF3-19, pIF14-23 and pIF4-4 were sequenced (see section 2.2.2.4).

3.4.1 Sequences of ACLSV

The genomic cDNA of ACLSV in pIF3-15 was assembled and analyzed. The corresponding virus was designed ACLSV isolate 38/85A. The genome of ACLSV 38/85A, excluding the polyadenylated (polyA) tail, consists of 7561 nucleotides (nts). Besides the 5' and 3' untranslated regions (UTRs), there are three open reading frames (ORFs) in the genome, i.e. ORF1, ORF2 and ORF3 (Figure 3.6). The 5' UTR consists of 149 nts, the position in the genome is from (nucleotide) nt 1 to 149. The ORF1 consists of 5658 nts, encoding the RNA-dependent RNA polymerase (RdRp), is located at position from nt 150 to 5807. The ORF2 consists of 1383 nts, encoding the movement protein (MP), is located at position from nt 5719 to 7101. The ORF3 consists of 582 nts, encoding the (coat protein) CP unit, is located at position from nt 6785 to 7366. The 3' UTR consists of 195 nts, the position in the genome is from nt 7367 to 7561. The ORF1 and ORF2 overlap from nt 5719 to 5807 (89 nts in length). ORF2 and ORF3 overlap from nt 6785 to 7101 (317 nts in length).

The genomic cDNA in pIF3-19 corresponded to the genome of ACLSV isolate 38/85B. The genome organization of ACLSV 38/85B is shown in Figure 3.6. The genome consists of 7544 nts excluding the polyA tail. The 5' UTR consists of 150 nts, the position in the genome is from nt 1 to 150. The ORF1 consists of 5652 nts, encoding the RdRp, and is located at position from nt 151 to 5802. The ORF2 consists of 1380 nts, encoding the MP, is located from nt 5714 to 7093. The ORF3 consists of 582 nts, encoding a unit of CP, is located at position from nt 6777 to 7358. The 3' UTR consists of 186 nts, the position in the genome is

from nt 7359 to 7544. The ORF1 and ORF2 overlap from nt 5714 to 5,802 (89 nt in length). The ORF2 and ORF3 overlap from nt 6777 to 7093 (317 nts in length).

The genomic cDNA in pIF14-23 corresponded to the genome of ACLSV isolate (36)/88. The genome organization of ACLSV (36)/88 is shown in Figure 3.6. The genome consists of 7549 nts excluding the polyA tail. The 5' UTR consists of 148 nts, the position in the genome is from nt 1 to 148. The ORF1 consists of 5661 nts, encoding the RdRp, is located at position from nt 149 to 5809. The ORF2 consisted of 1383 nts, encoding the MP, is located at postion from nt 5721 to 7103. The ORF3 consists of 582 nts, encoding a unit of CP, is located at position from nt 6787 to 7368. The 3' UTR consists of 181 nts, the position in the genome started from nt 7369 to 7549. The ORF1 and ORF2 overlap from nt 5721 to 5809 (89 nts in length). The ORF2 and ORF3 overlap from nt 6787 to 7103 (317 nts in length).

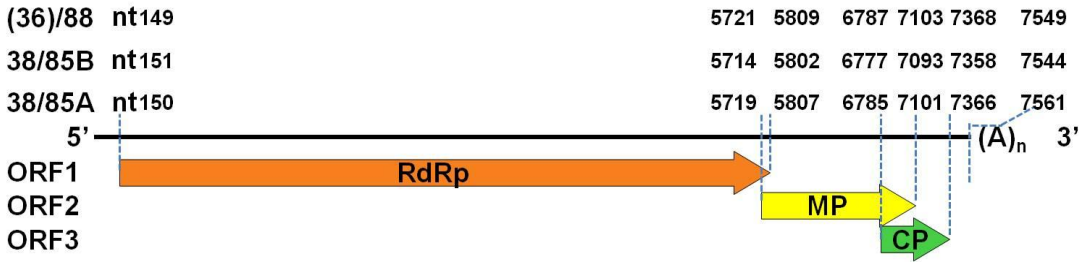

Figure 3.6 The genome organizations of the three obtained ACLSV isolates. The genome is made up of three open reading frames (ORFs), encoding RdRp, MP and CP respectively. Heterogeneity of the genomic sequences of the three isolates of ACLSV 38/85A (in pIF3-15), 38/85B (in pIF3-19) and (36)/88 (in pIF14-23) is described.

The three obtained ACLSV genomes from the infectious cDNA clones shared identities between 77.0% and 77.5% among each other. ACLSV (36)/88 (pIF14-23) shared identity of 77.0% with either ACLSV 38/85A or 38/85B. ACLSV 38/85A and 38/85B shared an identity of 77.5% with each other.

When aligned with the 18 sequences of complete ACLSV genomes published in NCBI, the sequence identities ranged from 66.5% to 79.4%. The 18 published sequences were: AB326223, AB326224, AB326225, D14996, EU223295, HE980332, JN634760, JN634761, KC935954, KC935955, KC935956, KJ522693, KM207212, KU870524, KU870525, M58152, NC_001409 and X99752. ACLSV 38/85A (pIF3-15) shared identities of 67.2%-78.5 with the 18 sequences, ACLSV 38/85B (pIF3-19) had identities of 68.1%-79.4%, and ACLSV (36)/88 (pIF14-23) shared identities of 66.5%-79.1%.

The sequences of ACLSV 38/85A and ACLSV 38/85B originally from the same sample were compared using MegAlign (DNASTAR Inc.). The genomes shared an identity of 77.5% with

each other. Their 5' UTR sequences were of 87.3% identical, their RdRp genes showed 76.1% identity, their MP genes 80.9% identity, and the CP genes 85.2% identity. The 3' UTR sequences were of 75.9% identical.

A phylogenetic tree was constructed for the three obtained ACLSV isolates of 38/85A, 38/85B and (36)/88, and the 18 sequences of complete ACLSV genome published in NCBI using MEGA5 (Figure 3.7). The tree contained two major clusters. ACLSV 38/85A, 38/85B and (36)/88 were in the same cluster. ACLSV (36)/88 was closely related to the ACLSV Bal 1 (X99752). The relationship of the ACLSV sequences does not indicate a geographic association.

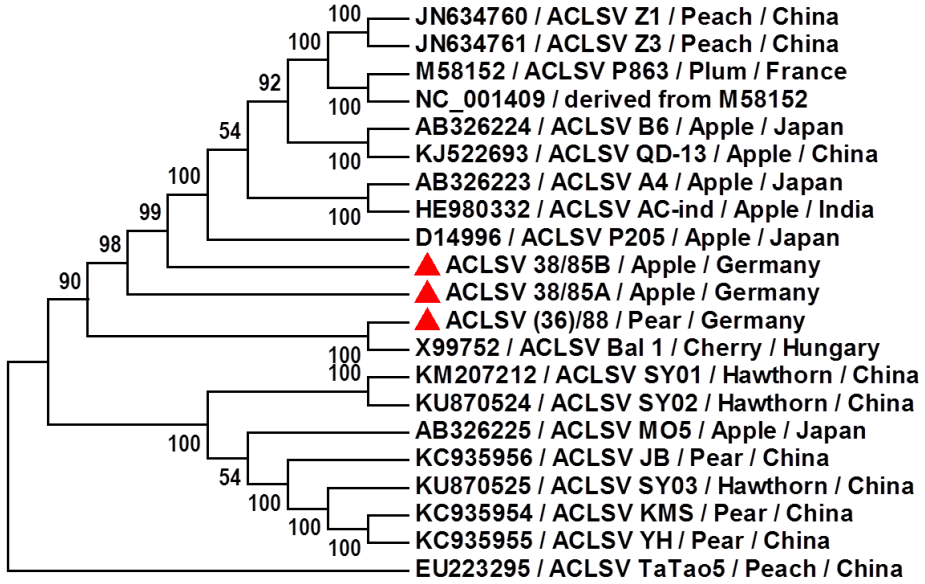

Figure 3.7 The phylogenetic tree based on complete genome sequences of ACLSV. The complete genome sequences used for analysis contained 18 published in NCBI and the sequences of ACLSV 38/85A (in pIF3-15), 38/85B (in pIF3-19) and (36)/88 (in pIF14-23). Red triangles indicate the ACLSV isolates obtained in the present work. The tree was generated using neighbor-joining method with bootstrap value of 1000.

3.4.2 Sequences of ASPV

The genomic cDNA in pIF4-4 was assembled and analyzed. The corresponding virus was ASPV isolate 40/87. The genome of ASPV 40/87, excluding the polyA tail, consists of 9270 nts. Besides the 5' and 3' UTRs, there are five ORFs in the genome, i.e. ORF1, ORF2, ORF3, ORF4 and ORF5 (Figure 3.8). The 5' UTR consists of 60 nts, the position in the genome is from nt 1 to 60. The ORF1 consists of 6546 nts, encoding the RdRp, is located at position from nt 61 to 6606. The ORF2 consists of 672 nts, encoding the triple gene block protein (TGB) 1, is located at position from nt 6705 to 7376. The ORF3 consists of 363 nts, encoding the TGB 2, is located at position from nt 7378 to 7740. The ORF4 consists of 228 nts,

encoding the TGB 3, is located at position from nt 7649 to 7876. The ORF5 consists of 1185 nts, encoding a unit of CP, is located at position from nt 7952 to 9136. The 3' UTR consists of 134 nts, the position in the genome is from nt 9137 to 9270. The ORF3 and ORF4 overlap from nt 7649 to 7740 (92 nts in length).

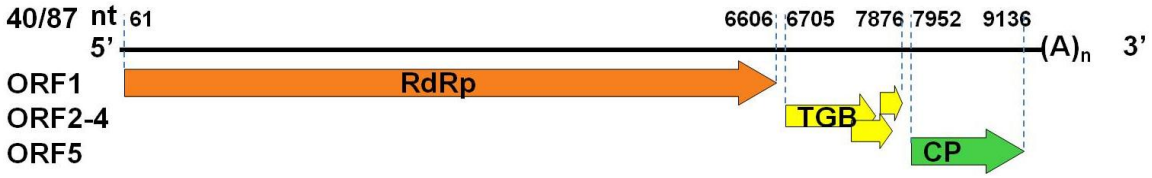

Figure 3.8 The genome organization of the obtained ASPV. ASPV 40/87 is in the clone pIF4-4. The genome consists of five open reading frames (ORFs), encoding RdRp, TGB proteins 1-3 and the CP. The nt position of each gene is shown above the diagram.

The complete sequence of ASPV 40/87 was aligned with the 12 complete sequences of ASPV published in NCBI. The 12 sequences were AB045371, EU095327, FR694186, JF946775, JF946772, KF319056, KF321966, KF321967, KF915809, KJ522472, KU308398, and LM999967. The sequence of ASPV 40/87 shared identities of 71.2% to 79.4% with the 12 published sequences. The highest identity of 79.4% was with KF915809 (ASPV isolate YT from apple in China), whilst the lowest identity of 71.2% was with EU095327 (ASPV isolate PR1 from pear in China).

A phylogenetic tree was constructed based on the sequences of ASPV 40/87 and the other 12 sequences in NCBI (Figure 3.9). From the tree, it was seen that ASPV 40/87 was close to the ASPV Hannover (KF915809) and ASPV YT (KF321967). ASPV Hannover was from *M. domestica* at JKI, Dossenheim, Germany. The ASPV YT was found in apple in China. The relationship of the ASPV viral sequences does not indicate a geographic association.

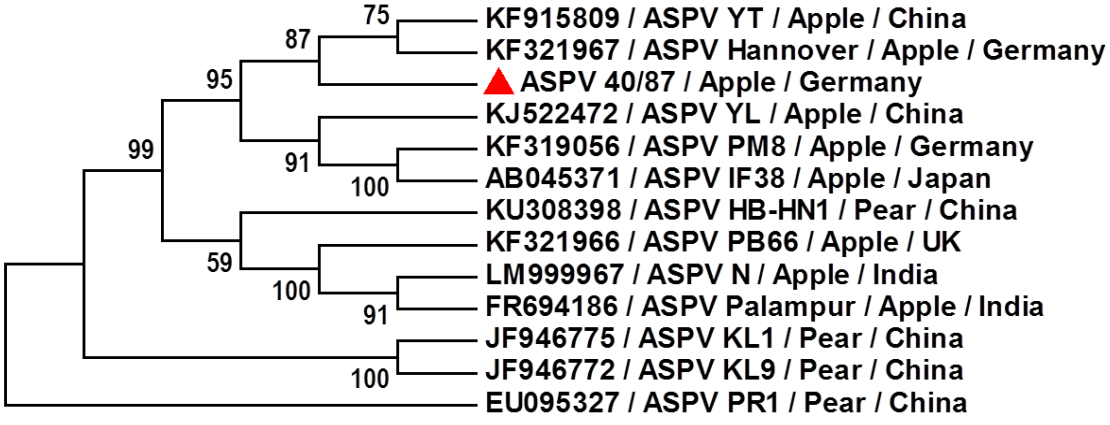

Figure 3.9 The phylogenic tree based on complete genome sequences of ASPV. The sequences used for analysis contained 12 sequences published in NCBI and the sequence of ASPV 40/87 (in pIF4-4). Red triangle indicates ASPV 40/87. The tree was constructed using a neighbor-joining method with bootstrap value of 1000.

3.5 Labeling of ACLSV isolate 38/85 with marker genes

3.5.1 Construction of the plasmids containing marker genes

To label the MP of ACLSV 38/85B at the C-terminus, the gene of Emerald-GFP (EmGFP) was fused to the 3' end of MP gene using strategy A and B (see section 2.2.2.3). The resulted plasmids were pIF13-9, pIF18-2 and pIF25-7 (Figure 3.10). In the plasmid of pIF13-9, the overlapping part (nt 8109-8422) of MP and CP genes and a possible promoter (nt 7805-8108) of CP in pIF3-19 was duplicated, flanking the insert of EmGFP (nt 7091-7804). The plasmid of pIF18-2 was modified from the pIF13-9 by substituting the CP and a possible promoter in pIF3-15 (nt 7805-8473) for CP and the possible promoter in pIF13-9. The plasmid of pIF25-7 was constructed by substituting the MP:EmGFP in pIF13-9 (nt 5808-7809) for the MP of ACLSV 38/38A in pIF3-15.

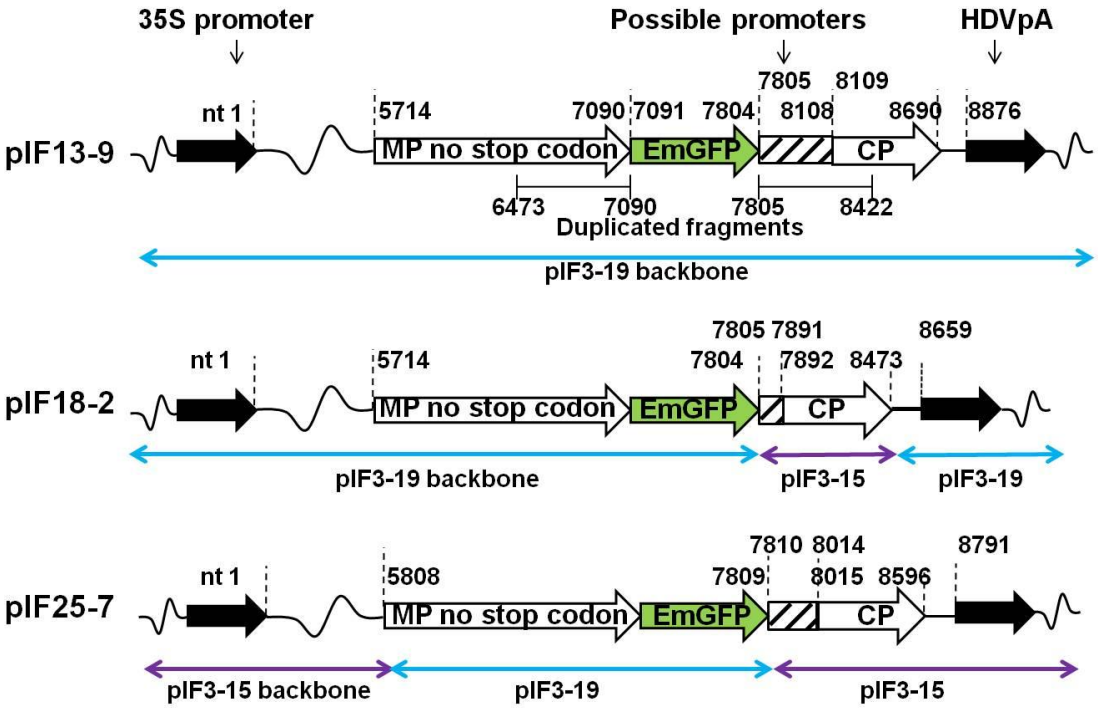

Figure 3.10 The labeling of ACLSV MP at C-terminus with EmGFP. The constructed plasmids were pIF13-9, pIF18-2 and pIF25-7. The clones pIF3-15 and pIF3-19 were used as backbones for modification. Nt positions of genes are shown on the diagrams. The 35S promoter and the HDVpA of pV297 vector are shown in black arrows.

To label the CP at the N-terminus, the gene of mCherry was fused to the 5' end of CP gene using strategy A and B (see section 2.2.2.3). The resulting plasmids were pG11-15, pIF23-1 and pIF24-6 (Figure 3.11). On plasmid pG11-15, the overlapping part of MP (nt 6777 – 7093) and CP genes (nt 8106 – 8422) and a possible promoter for CP (nt 7094 – 7397, identical to nt 6473 – 6776 of MP) was duplicated, flanking the insert of mCherry gene (without stop codon). The plasmid of pIF23-1 was constructed by substituting the promoter:mCherry:CP of pG11-15 (nt 7102 – 8695 in pIF23-1, nt 7094 – 8187 in pG11-15) for the CP in pIF3-15. The plasmid of pIF24-6 was constructed by substituting partial MP in pIF3-15 for the partial MP in pG11-15 (nt 5803 – 7096).

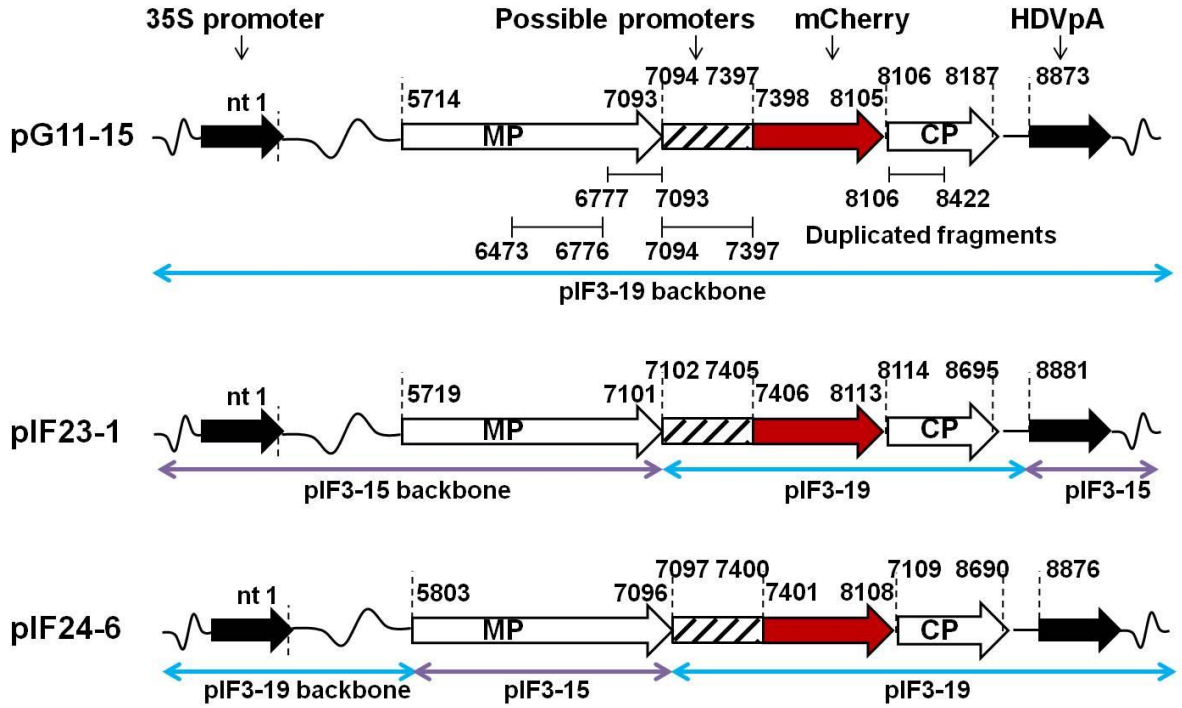

Figure 3.11 The labeling of ACLSV CP at N-terminus with mCherry. The obtained plasmids were pG11-15, pIF23-1 and pIF24-6. The clones pIF3-15 and pIF3-19 were used as backbones for modification. Nt positions of genes are shown on the diagrams. The genes of the 35S promoter and the HDVpA of vector pV297 are shown in black arrows.

To label the CP at the C-terminus, the gene of mCherry or iLov was fused to the 3' end of CP gene using Strategy C (see section 2.2.2.3). The resulting plasmids were pIF16-1, pIF20-16 and pIF27-10. The plasmid pIF16-2 was constructed by inserting a mCherry gene between the 3' end of CP gene and the 3' UTR in pIF3-19. The insertion site was in front of the stop codon of the CP. The pIF20-16 was modified from pIF16-2 by inserting a linker of $(EAAAK)_4$ between CP and mCherry. The plasmid pIF27-10 was modified from pIF16-2 by substituting the iLov gene for the mCherry gene (Figure 3.12).

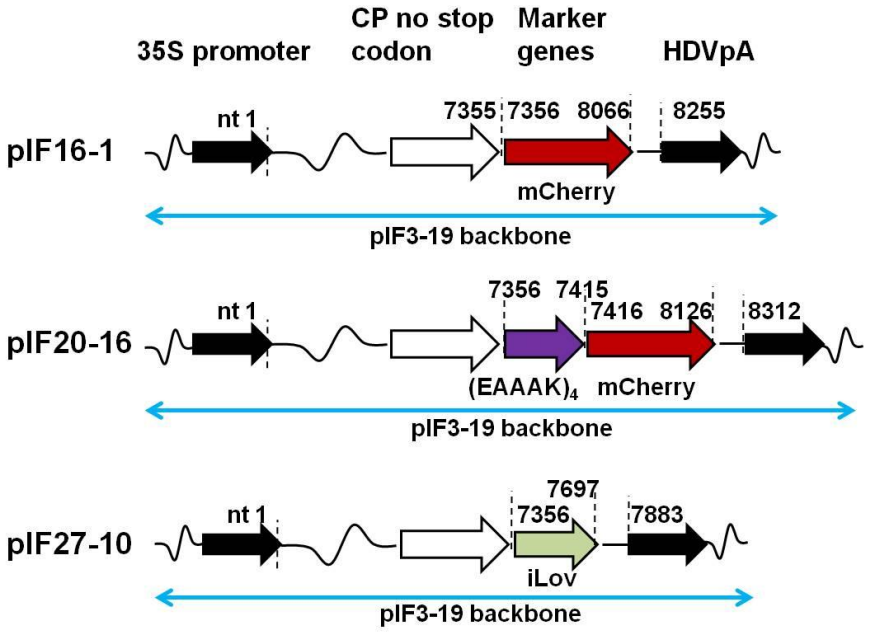

Figure 3.12 The labeling of ACLSV CP at C-terminus with mCherry or iLov. The obtained plasmids were pIF16-1, pIF20-16 and pIF27-10. The clone pIF3-19 was used as backbone for modification. Nt positions of genes are shown on the diagrams. The 35S promoter and the HDVpA of pV297 vector are shown in black arrows.

3.5.2 Detection of marker genes in constructed plasmids and transformed *A. tumefaciens*

The existence of the marker genes in the constructed plasmids was confirmed by PCR (see section 2.2.1.3) using primers #050/#047 (Figure 3.13A). The primer #050 binding site was nt 5829 – 5849 in the genomic cDNA of ACLSV 38/85B (in pIF3-19), whilst the primer #047 binding site was nt 8831 – 8860. The expected bands contained partial viral fragments and the complete marker genes of EmGFP, mCherry or iLov. The PCR products of expected sizes of 3032, 2815, 2942, 3029, 3032, 3032, 2411, 2468 and 2039 bp were amplified from pIF13-9, pIF18-2, pIF25-7, pG11-15, pIF24-6, pIF23-1, pIF16-1, pIF20-16 and pIF27-10, respectively. From pIF3-19 (the clone used as backbone for labeling), a product of 1700 bp was generated. No PCR product was amplified using pV297 as template.

Before performing agroinoculation, the marker genes were also detected in the inocula (agrobacteria) as described above. The same result was obtained. (Figure 3.13B).

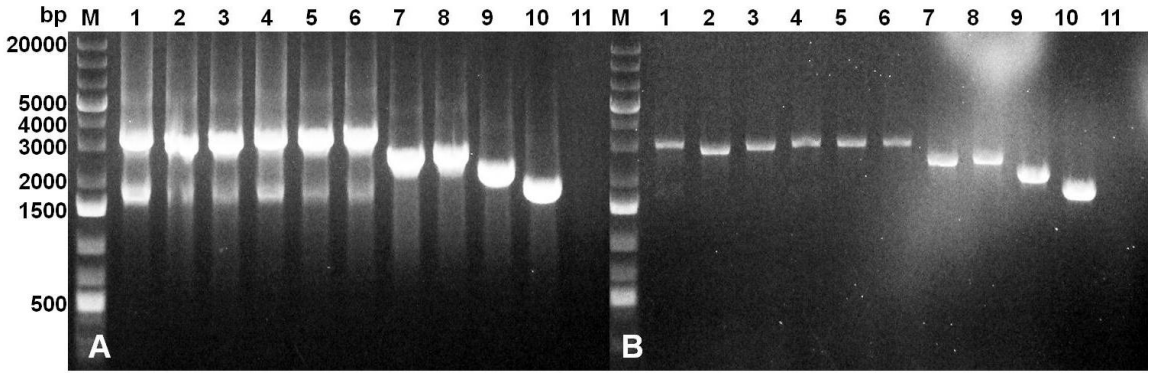

Figure 3.13 PCR detection of marker genes before performing agroinoculation. The existence of marker genes in the constructed plasmids (A) and the transformed *A. tumefaciens* (B) was confirmed by PCR. Lanes 1 to 9: pIF13-9, pIF18-2, pIF25-7, pG11-15, pIF24-6, pIF23-1, pIF16-1, pIF20-16 and pIF27-10, respectively. Lane 10: pIF3-19. Lane 11: pV297 vector. M: GeneRuler 1 kb plus DNA ladder. The sizes of these bands were 3032, 2815, 2942, 3029, 3032, 3032, 2411, 2468, 2039, and 1700 bp, respectively.

3.5.3 Agroinoculation of *N. occidentalis* 37B with labeled plasmids

The agroinoculation of *N. occidentalis* 37B with the constructed plasmids (pIF13-9, pIF18-2, pIF25-7, pG11-15, pIF24-6, pIF23-1, pIF16-1, pIF20-16 and pIF27-10) was performed four times. The results, however, were different; mainly two results were observed. One result (see section 3.5.3.1) was that pIF13-9 and pG11-15 caused systemic infection in agroinoculated plants, and this result was observed in three independent trials. Therefore one construct (pIF13-9), using EmGFP fused to the C-terminus of the ACLSV MP and one construct (pG11-15), using mCherry fused to the N-terminus of the CP resulted in systemic ACLSV infection. The other result (see section 3.5.3.2) was that pG11-15 and pIF25-7 caused systemic infection in test-plants, and this was observed in a trial at winter time. Furthermore, the inoculated and noninoculated leaves were examined for viral infection.

3.5.3.1 First agroinoculation of *N. occidentalis* 37B

After agroinoculation with the constructed plasmids (pIF13-9, pIF18-2, pIF25-7, pG11-15, pIF24-6, pIF23-1, pIF16-1, pIF20-16 and pIF27-10), the total nucleic acid of the test-plants were extracted using silica capture method (see section 2.2.1.1). The expected marker genes were detected in the extracted nucleic acids by RT-PCR using primers #55/#80 (see sections 2.2.1.2 and 2.2.1.3). The binding site of #55 was nt 7335 – 7355 of the ACLSV genomic cDNA in pIF3-19 (CP gene), whilst the binding site of #80 was nt 6438 – 6456 (MP gene). The vector pV297 and the infectious clone of pIF3-19 were the controls.

Four days after agroinoculation with pIF13-9 and pG11-15, the marker genes in the test-plants were detected for the marker genes by RT-PCR using primers #55/#80. The PCR products of

expected sizes of 2250 and 2247 bp were generated from pIF13-9- and pG11-15-inoculated samples respectively. In addition, products of unexpected size of around 1000 bp were generated too (Figure 3.14A and C). No band was amplified from pV297-inoculated samples.

Seven days after agroinoculation, PCR products of 918 bp were amplified from pIF13-9-inoculated plants (Figure 3.14B), which were identical to that amplified from the pIF3-19-inoculated (the unmodified infectious clone of ACLSV) plants. The result of pG11-15-inoculated plants was similar (Figure 3.14D). No band was amplified from pV297-inoculated samples.

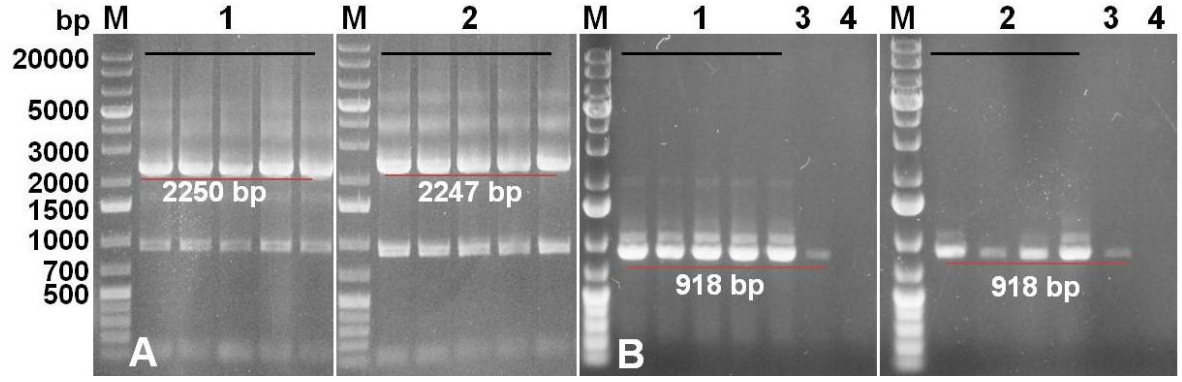

Figure 3.14 RT-PCR detection of marker genes in agroinoculated plants. Five or four plants of *N. occidentalis* 37B were agroinoculated with the constructed clones. The detection was performed four day (A) and seven days (B) after agroinoculation, respectively. Lanes 1: pIF13-9. Lanes 2: pG11-15. Lanes 3: pIF3-19 (wild type). Lanes 4: pV297 (negative control). Lanes M: GeneRuler 1 kb plus DNA ladder. The expected bands were indicated by red lines.

Around nine days after agroinoculation with pIF13-9 and pG11-15, the test-plants started to develop symptoms. The symptoms were similar to those caused by pIF3-15 or pIF3-19 (the unmodified infectious clones of ACLSV). Using Anti-CP antibody, the proteins of ca. 22 kDa were detected in the symptomatic plants (Figure 3.15). Viral particles of ACLSV were detected in the symptomatic plants by ISEM (data not shown).

Agroinoculation of pIF18-2, pIF25-7, pIF24-6, pIF23-1, pIF16-1, pIF20-16 and pIF27-10 did not cause infection on any test-plants. The fragments of marker genes cannot be detected by RT-PCR. No viral fragments were detected by RT-PCR.

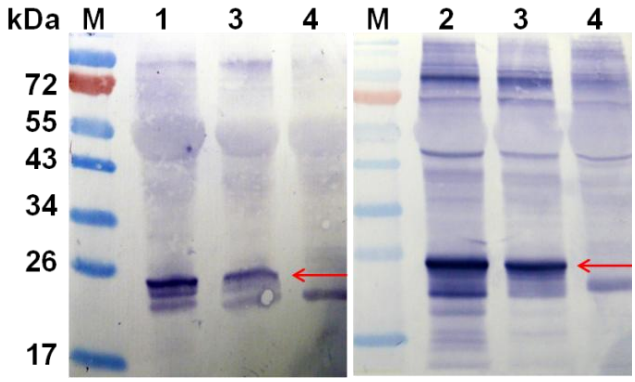

Figure 3.15 Detection of ACLSV CP in agroinoculated plants by western blot. Around nine days after agroinoculation, the ACLSV CP was detected in the inoculated N. occidentalis 37B. The used antiserum was Anti-CP antibody. The test-plants were inoculated with: pIF13-9 (lane 1), pG11-15 (lane 2), pIF3-19 (wild type) (lanes 3) and pV297 (negative control) (lanes 4). Red arrows indicate the CPs of ca. 22 kDa.

3.5.3.2 Second agroinoculation of *N. occidentalis* 37B

3.5.3.2.1 Infection in inoculated leaves

In the inoculated leaves, the fused genes of EmGFP, mCherry and iLov can be detected by RT-PCR. For detection, total nucleic acids of the inoculated leaves were extracted 16 days after agroinoculation, followed by generation of cDNAs (see sections 2.2.1.1 and 2.2.1.2). The cDNAs were used as templates in the detection of the marker genes using primers #050/#047. The primer #050 binding site was nt 5829 – 5849 in the genomic cDNA of ACLSV 38/85B (in pIF3-19), whilst the primer #047 binding site was nt 8831 – 8860. To avoid potential contamination of plasmid DNA or T-DNA from agrobacteria, the total nucleic acids were directly used as templates in PCR amplification too. From the cDNA templates, PCR products of expected sizes of 3032, 2815, 2942, 3029, 3032, 3032, 2411, 2468, 2039 and 1700 bp were generated for pIF13-9, pIF18-2, pIF25-7, pG11-15, pIF24-6, pIF23-1, pIF16-1, pIF20-16, pIF27-10 and pIF3-19, respectively (Figure 3.16A). The band pattern was similar to that in Figure 3.13 (see section 3.5.2). When total nucleic acids were used as templates in PCR, no PCR product was generated. Therefore, the PCR products of the expected size resulted from the expression of the modified ACLSV genome, but not from DNA contamination.

The PCR detection of the marker genes was repeated once 36 days after agroinoculation by the method described. From the cDNA templates, products of 3032, 2815, 1700, 1700, 3032, 3032, 2411, 2468, 2039 and 1700 bp were amplified (Figure 3.16B). No product was amplified from the total nucleic acids.

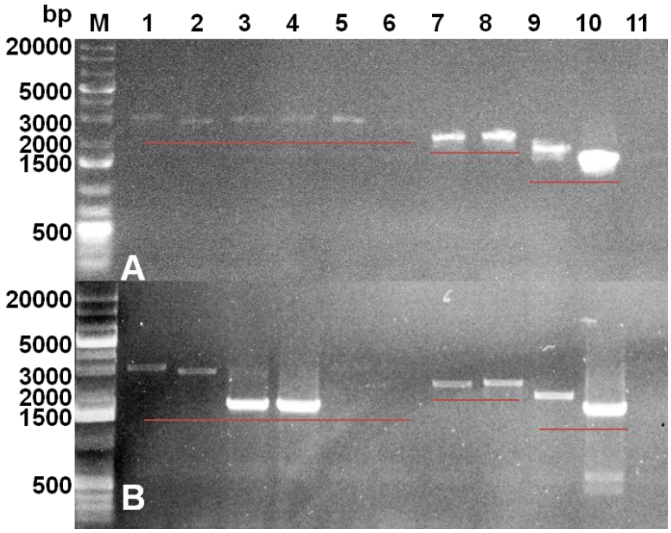

Figure 3.16 RT-PCR detection of marker genes in agroinoculated leaves. The detection was performed 16 days (A) and 36 days (B) after agroinoculation, respectively. The samples (*N. occidentalis* 37B) were agroinoculated with pIF13-9 (Lane 1), pIF18-2 (Lane 2), pIF25-7 (Lane 3), pG11-15 (Lane 4), pIF24-6 (Lane 5), pIF23-1 (Lane 6), pIF16-1 (Lane 7), pIF20-16 (Lane 8), pIF27-10 (Lane 9), pIF3-19 (wild type) (Lane 10) and pV297 (negative control) (Lane 11) respectively. The sizes of the fragments in each lane were 3032, 2815 (1700 bp in B), 2942 (1700 bp in B), 3029, 3032, 3032, 2411, 2468, 2039 and 1700 bp, respectively.

The protein expression of the constructed plasmids was determined by western blot 36 days after inoculation (see section 2.2.5). The antibodies of Anti-CP, Anti-MP and Anti-mCherry were used for detection of ACLSV CP, MP and the fluorescent protein of mCherry, respectively (Table 2.6).

The results (Figure 3.17) indicated that proteins of expected size of ca. 22 kDa (the CP of ACLSV) were detected in the leaves agroinoculated with pIF23-1 and pIF3-19. In the plants agroinoculated with pIF3-19 only proteins of ca. 50 kDa (the MP of ACLSV) were detected. A band of ca. 26 kDa was seen from the leaves agroinoculated with pIF3-19 using the anti-mCherry antibody.

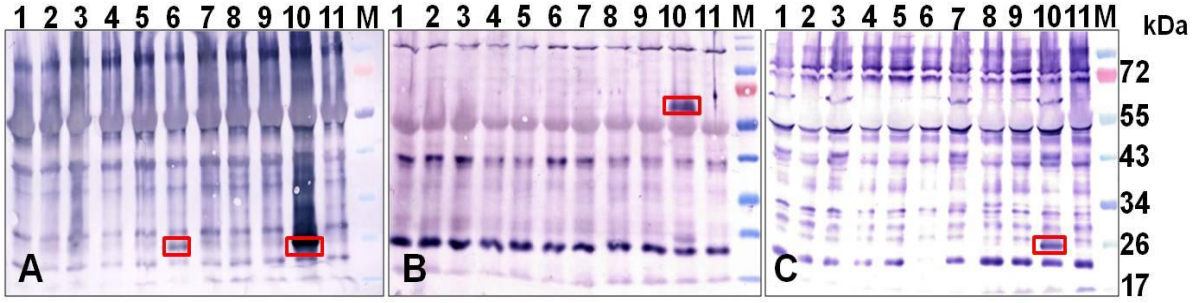

Figure 3.17 Detection of viral proteins in agroinoculated leaves by western blot. The antibodies were Anti-CP (A), Anti-MP (B) and Anti-mCherry (C), respectively. The proteins were extracted from *N. occidentalis* 37B agroinoculated with pIF13-9 (Lane 1), pIF18-2 (Lane 2), pIF25-7 (Lane 3), pG11-15 (Lane 4), pIF24-6 (Lane 5), pIF23-1 (Lane 6), pIF16-1 (Lane 7), pIF20-16 (Lane 8), pIF27-10 (Lane 9), pIF3-19 (Lane 10) and pV297 (Lane 11), respectively. The target bands are indicated by red boxes.

3.5.3.2.2 Infection in noninoculated leaves

Sixteen days after the agroinoculation, noninoculated leaves of test-plants were detected by RT-PCR using primers #050/#047, viral fragments of ca. 1700 bp were amplified from the plants agroinoculated with pG11-15 and pIF3-19 (Figure 3.18A). Thirty-six days after inoculation, the detection was performed again. The same result was obtained as shown in Figure 3.18B. No product was amplified from the total nucleic acids of plants agroinoculated with pIF13-9, pIF18-2, pIF25-7, pIF24-6, pIF23-1, pIF16-1, pIF20-16 and pIF27-10.

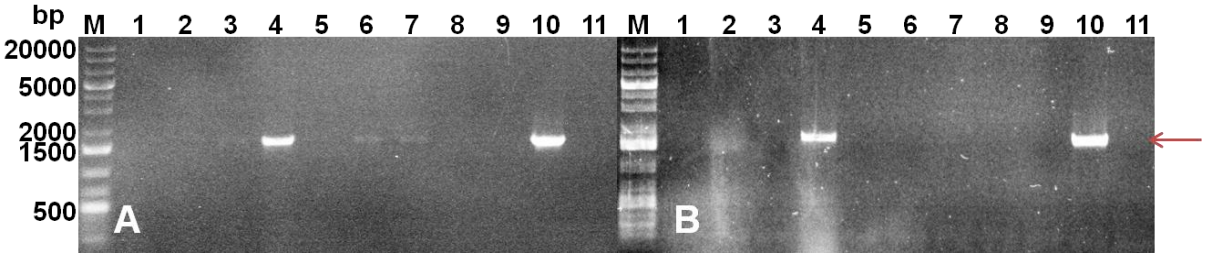

Figure 3.18 RT-PCR detection of marker genes in noninoculated leaves. The detection was performed 16 days (A) and 36 days (B) after agroinoculation, respectively. The test-plants of *N. occidentalis* 37B were agroinoculated with pIF13-9 (lane 1), pIF18-2 (lane 2), pIF25-7 (lane 3), pG11-15 (lane 4), pIF24-6 (lane 5), pIF23-1 (lane 6), pIF16-1 (lane 7), pIF20-16 (lane 8), pIF27-10 (lane 9), pIF3-19 (wild type) (lane 10) and pV297 (negative control) (lane 11), respectively. The target bands are indicated by red arrow.

The clones of pG11-15 and pIF3-19 caused systemic infection on N. occidentalis 37B plants around 12 days after agroinoculation, whilst pIF25-7 caused symptoms 40 days after agroinoculation. The symptoms caused by pG11-15 and pIF25-7 were similar to those caused by pIF3-19 (the wild-type control).

In summary, the constructs of pIF13-9 and pG11-15 can cause systemic symptoms on agroinoculated *N. occidentalis* 37B. The symptoms were similar to those caused by pIF3-19 (the wild type). However, marker genes cannot be detected in the infected plants. The other constructs (pIF18-2, pIF25-7, pIF24-6, pIF23-1, pIF16-1, pIF20-16 and pIF27-10) did not cause any symptoms on agroinoculated *N. occidentalis* 37B, but the marker genes can be detected in inoculated leaves, not in noninoculated leaves, by RT-PCR in 36 days. However the expression of viral proteins or fluorescent proteins needs to be further investigated.

4. Discussion

4.1 Investigation of different cloning techniques to construct full-length cDNA clones of ACLSV and ASPV

Different methods were used in the cloning of ACLSV and ASPV in the present work. These methods included circular polymerase extension cloning (CPEC), Gibson assembly and In-Fusion cloning. The protocols of these methods were optimized during the work. Several considerations were taken into account. First, in all techniques to amplify the full-length genomic cDNA of the viruses, the primers should not contain any extension part which is homologous to the ends of the linearized vector. Otherwise, failed amplification or low yield of the viral genomic cDNAs can be expected during the PCR. Second, in the linearization of pV297 by PCR, the amount of the plasmid template should be as low as possible, such as 0.1 ng in 50 µl. Furthermore the PCR product of linear pV297 should be digested with *Dpn*I which cleaves only *E. coli* Dam methylase-methylated plasmid DNA, but does not cleave the PCR product since it is not methylated. With successful control of the template amount and performance of the *Dpn*I digestion, the number of false colonies can be greatly reduced to a very low level or even be totally eliminated. This made the selection of positive colonies more efficient. Finally gel extraction was recommended for the purification of target fragments of the insert and the vector. Gel purification can increase the purity of target fragments, thus reducing unexpected fusion during Gibson assembly and In-Fusion cloning or the unspecific annealing of insert and vector during CPEC.

It was found that In-Fusion cloning worked more efficient than Gibson assembly and CPEC in the present work. On one hand, the number of obtained full-length cDNA clones by In-Fusion cloning was higher than that by CPEC or Gibson assembly. For example, in construction of full-length clones of ACLSV (27)/85, three clones were obtained by In-Fusion, one was obtained by CPEC and Gibson assembly respectively (Table 3.1). On the other hand, the In-Fusion cloning was exacter than the CPEC and Gibson assembly. The cloning parts of pV297 vector and viral genomic cDNAs of all the obtained clones were sequenced, and the

results indicated that all the clones constructed by In-Fusion have exact cloning parts of the 15 bp, whilst the clones constructed by CPEC or Gibson have extra non-viral nucleotides introduction or reduction at the ends of viral genomic cDNAs (data not shown). Based on this hypothesis, In-Fusion was decided the main method in the present work, and it worked well throughout the whole project.

Compared with cloning systems that depend on several cloned or PCR amplified cDNA fragments and their fusion, In-Fusion cloning has an advantage of simplicity and is ligation independent. In previous work of construction of infectious full-length cDNA clones of ACLSV P-205, restriction digestion and a ligation-based method (Satoh et al. 1999), or an *in vivo* yeast homologous recombination system (Youssef et al. 2011a) were used. The restriction digestion and ligation-based method requires unique and specific sites in both the insert and the vector and the ligation steps are usually inefficient (Stevenson et al. 2013). The *in vivo* yeast system has the advantage of avoiding ligation, however, the strategy requires a ternary yeast-*E. coli*-*A. tumefaciens* vector. Multiple transformation steps in *E. coli*, yeast and *A. tumefaciens* increase the risk of mutations of the cDNA clones. In comparison with the restriction digestion and ligation-based method and the *in vivo* yeast system for ACLSV full-length clone construction, the In-Fusion cloning is simpler as specific restriction sites in the insert and the vector are not required, and inefficient fusion steps and a ternary vector are avoided. The overlapping 15 nucleotides between vector and viral insert can be exactly cloned by In-Fusion. This was confirmed by sequencing for the cloning sites of the four ACLSV full-length clones pIF3-12, pIF3-14, pIF3-15 and pIF3-19, and the two ASPV full-length clones pIF4-4 and pIF4-16 generated in this work. Extra non-viral nucleotides might significantly reduce virus infectivity (Hans et al. 1992; Janda et al. 1987). The lack of infectivity for the ACLSV clones pIF3-12 and pIF3-14 was not due to problems at the cloning sites. For clone pIF3-14 a frameshift in the RNA-dependent RNA polymerase region was determined and assumed to be the reason for lack of infectivity. For example, Ishikawa et al. (1986) found that frame-shift mutants with an intact 130K but a defective 180K protein (putative TMV replicase) gene of TMV were not infectious. Clone pIF3-12 showed considerable variation in the 3' and 5' terminal nucleotides in comparison to the two infectious clones. Either this variation or possible unexpected mutation(s) during PCR, or during *E. coli* or *A. tumefaciens* transformation steps might explain lack of infectivity for this construct.

The infectious full-length cDNA clone of *Papaya leaf distortion mosaic virus* (PLDMV) was recently constructed using In-Fusion cloning (Tuo et al. 2015). PLDMV belongs to the genus

Potyvirus and its genome comprises 10,153 nucleotides excluding the polyA tail. Therefore, In-Fusion cloning has the potential of application to most filamentous RNA viruses.

Another major advantage of using full-length PCR to produce the ACLSV (ca. 7.5 kb) and ASPV (9.5 kb) genome is its independence of the highly variable genome of ACLSV and ASPV isolates. Their conserved terminal end sequences allow design and use of primers which can be applied to a wide range of isolates if not all ACLSV or ASPV strains. In NCBI there were other 18 complete sequences of ACLSV isolates, in comparison with ACLSV 38/85A, 38/85B and (36)/88, although identity of 66.5 to 79.4% was observed between theses ACLSV isolates (see section 3.4.1), the 5' and 3' termini are highly conserved. It was seen that 12 of 21 isolates (including the three in present work) have the identical sequences of first 32 nts at their 5' termini, except for four isolates (accession no. KC935954, KC935955, KM202212 and KU870525) were not reported with the complete first 32 nts. Otherwise this number may increase. Moreover 18 of 21 isolates have the identical sequences of 34 nts at 3' termini, except for the last 34 nts of one isolate (accession no. HE980332) were not reported in GenBank. It is sure that 32 nts or 34 nts were long enough for primer design. Similarly except for the sequences of 6 isolates (accession no. LM999967, JF946775, JF946772, FR694186, KJ522472 and EU095327) were not reported, the other 7 of 13 ASPV isolates (including ASPV 40/87) have the identical sequences of 27 nts at their 5' termini, whilst 8 isolates have the identical sequences of 25 nts at 3' termini. The conserved sequences are long enough to be used for designing primers too. This approach can be applied to other viruses that show high variability but with conserved terminal sequences (Yoon et al. 2014; Youssef et al. 2011b).

4.2 Agroinoculation of herbaceous and woody plants

Chenopodium quinoa is a useful experimental host of ACLSV (Myrta et al. 2011). It has played an important role in propagation and purification of ACLSV (German et al. 1990; Yoshikawa and Takahashi 1988) or as assay host of ACLSV (Dhir et al. 2013). In the present work, the infectious cDNA clones of pIF3-15, pIF3-19 and pIF14-23 were agroinoculated to *C. quinoa.* However, no infection of any ACLSV isolate was established in *C. quinoa*. The reason of the failed infection was unknown. We first examined the virus isolates based on sequence data of the obtained genomic cDNAs (see section 3.4.1). It is said that combination of two amino acids (Ala^{40} - Phe^{75} or Ser^{40} - Tyr^{75}) in CP is necessary for infection to *C. quinoa* by mechanical inoculation (Yaegashi et al. 2007). The ACLSV isolates 38/85A (pIF3-

15), 38/85B (pIF3-19) and (36)/88 (pIF14-23) had the combination of amino acid sequences of Ser^{40}-Tyr^{75}, Ala^{40}-Phe^{75}, and Ser^{40}-Tyr^{75}, respectively. Based on this knowledge, the CP amino acids of the tested ACLSV isolates were not the reason for failed infection in *C. quinoa*. Other possibilities were that the *C. quinoa* may preform structural barriers or toxic compounds that confine infection establishment or the pathogen (transformed agrobacteria or the nucleic acids of ACLSV) perishes before the establishment of the infection (Hammond-Kosack and Jones 2015), but there was no available evidence in publications supporting the hypotheses as so far.

The agroinoculation experiments of apple seedlings were successful and highly efficient. The overall survival rate of the infiltrated seedlings was between 21% (5/23, pIF3-19, Figure 3.5A) and 93% (14/15, pIF3-15, Figure 3.5B-C). The PCR positive rate was between 21% (3/14, pIF3-15) and 100% (11/11, pIF3-19) (Figure 3.5B). Compared with the mechanical agroinoculation of 6-month apple seedlings, in which the establishment of infection of ACLSV infectious clones failed mostly (at least 5 failure in 5 independent experiments), but once we observed a PCR-positive rate of 2.5% (2/80) (data now shown in the work), the result of the vacuum infiltration above was satisfactory. Moreover, it is reported that biolistic inoculation is an efficient method of inoculation of viral RNAs (total RNAs from infected tissues or virus RNAs from purified virus) to apple seedlings (Yamagishi et al. 2010). For example, in the case of ACLSV, it showed that 6 out of 7 plants (86%) were infected, the efficiency of the biolistic inoculation was comparable to the vacuum infiltration described in the present work. However, given the consumption of gold particles and other consumables, the vacuum infiltration is scalable and low-cost. Although agroinoculated full-length viral cDNA clones could cause false positives in PCR detection, due to systemic spread of the bacterium in host species (Cubero et al. 2006), infections of ACLSV in woody plants were confirmed by ISEM in addition to PCR detection.

The vacuum infiltration parameters may influence the survival rates of infiltrated seedlings. With the vacuum parameters of treatment 1 the survival rates of 21% (5/23) and 51% (18/35) were observed for wounded and unwounded seedlings, respectively (Figure 3.5A). While the much lower vacuum used in treatment 2 and 3 resulted in survival rates above 66% (10/15, pIF3-19, Figure 3.5B). These results indicate that the high air pressure of 500 hPa may be more beneficial to the survival of apple seedlings. However, the effect of air pressure (strength of vacuum) on survival rate of infiltrated apple seedlings remained unclear. During vacuum infiltration, air is forced out of the intercellular spaces within the leaves through

stomata and possibly through wounding sites, and pressure difference forces the agrobacteria suspension into the leaves (Simmons et al. 2009). There is assumption that water injection of the air spaces in leaf tissue neither are injuring cell membranes nor are altering the cellular metabolism (MacDonald 1975). But it is known that if drop in pressure is too rapid (because of the power of the pump), or when the vacuum is suddenly broken, it can cause death of *Arabidopsis thaliana* plants after vacuum infiltration (Bechtold and Pelletier 1998). Moderate pressure reduction is used in food storage by vacuum cooling. It had positive effects on storage quality of iceberg lettuce by retaining tissue firmness (He et al. 2004).

The duration of vacuum may not greatly influence the success of agroinoculation of apple seedlings with the infectious clones as expected. In the vacuum infiltration 2, vacuum time of 2, 5 and 10 min was tested in the agroinoculation of wounded seedlings with pIF3-15 and pIF3-19 respectively (Figure 3.5B). In the agroinoculation with pIF3-15, the survival rates were respective 93% (14/15), 86% (13/15) and 80% (12/15) under the time conditions of 2, 5 and 10 min, whilst the corresponding PCR-positive rates were 21% (3/14), 23% (3/13) and 50% (6/12). The results indicated that the survival rates decreased from 93% (14/15) to 80% (12/15) with the increase of vacuum time from 2 to 10 min, and the PCR-positive rates rose up with the increase of the vacuum time. However, in the agroinoculation with pIF3-19, the results indicated that the survival rates increased from 66 (10/15) to 73% (11/15) with the prolongation of the vacuum time, and the PCR-positive rate also increased from 80% (8/10) to 100% (11/11). From these results we cannot confirm the influence of vacuum time with the survival rates of the infiltrated seedlings, and the longer duration of vacuum may be beneficial to the PCR-positive rates, but not obvious.

The wounding of cotyledons resulted in higher infection rates with the virus but this was only done in treatment 1. With wounding the PCR positive rate of 80% (4/5) for wounded seedlings was significantly higher than the 11% (2/18) for unwounded seedlings (Figure 3.5A). This result indicates that wounding of cotyledons considerably increased the efficiency of agroinoculation of the infectious full-length cDNA clones of ACLSV to woody plants. It has been reported that physical wounding of plants could significantly influence transformation efficiency (Norelli et al. 1996; Rashid et al. 1996). Crushing apple leaves by using nontraumatic forceps before inoculation resulted in a 10-fold increase in the amount of transformation when compared with the unwounded control (Norelli et al. 1996).

The cold pretreatment of apple seedlings might also influence the rates of infectivity. It is known that exposure of plants to low temperatures before inoculation may increase their

susceptibility to disease. For example, cold pretreatment of seeds of bean, pea, maize, or cucumber, in the early stages of germination prior to being exposed to infection by *Rhizoctonia solani* Kühn, increased their susceptibility (Schulz and Bateman 1969). This cold treatment apparently reduced the vigor of the plants, and thus, they were more sensitive to infection (Colhoun 1973). In another report using ACLSV inoculation of apple seedlings germinated in darkness at 4°C were inoculated by particle bombardment of total RNAs extracted from ACLSV-infected *C. quinoa*. After the biolistic inoculation, the seedlings were again placed in the dark at 4°C for 1-2 days. An efficiency of 86% (6/7) was observed (Yamagishi et al. 2010).

The virus isolate may influence the success of agroinoculation of the apple seedlings with the infectious clones. In the vacuum infiltration treatment 3, a PCR-positive rate of 25% (2/8) was observed for ACLSV (36)/88 (pIF14-23), which was lower than the 78% for ACLSV 38/85A and 87% for ACLSV 38/85B (Figure 3.5C) (see section 3.3.1). The low infection efficiency might be because ACSLV (36)/88 was from pear, and the test-plant was apple. The isolate (36)/88 might be better adapted to peach than to apple. It is reported that the lab strain of *Tobacco etch virus* (TEV) is adapted to tobacco, but when TEV was adapted to pepper, virulence increased, but was found to decrease in the tobacco host, suggesting a tradeoff in becoming more specialized (Bedhomme et al. 2012; Elena et al. 2008). ACLSV (36)/88 might be specialized in peach, resulting in the low infection rate in test-plants of apple seedlings.

The protocol of agroinoculation of infectious full-length ACLSV cDNA clones described here can be applicable to other woody hosts. ACLSV has a wide range of woody hosts such as apple, pear, quince, peach, plum, almond, apricot, cherry and hawthorns (Myrta et al. 2011; Yaegashi et al. 2011). It is reported that GF305 peach seedlings were infected with ACLSV P-205 by agroinfiltration (Youssef et al. 2011a), but at a low efficiency.

4.3 Phylogenetic analysis of ACLSV and ASPV

It is not unexpected that two variants of ACLSV were obtained from source 38/85. Single-strand conformation polymorphism analysis of the 3'-terminal 1.8 kb genomic cDNAs of 15 ACLSV isolates revealed that at least two to four sequence variants were found in declining apple trees, while in nondeclining trees, a major type sequence was dominant (Nakahara et al. 2011). Similarly coexistence of divergent viral variants of *Grapevine virus A* (GVA), type member of the genus *Vitivirus* in the family *Betaflexiviridae*, in individual grapevines is

observed too: the number of distinct haplotypes specific to RdRp and CP present in individual grapevines ranged from two to nine and two to eight, respectively (Alabi et al. 2014). In addition, it is known that ACLSV isolates show a high variability in their nt sequence with an overall identity between 76 and 82% (Adams et al. 2012; German-Retana et al. 1997). The three obtained ACLSV isolates of 38/85A, 38/85B and (36)/88 shared identities between 77.0% and 77.5% among each other. The identity numbers were in commonly observed variabilities between different strains of 76 and 82% (Adams et al. 2012; German-Retana et al. 1997).

Previous studies showed the genetic diversity of ASPV CP genes ranged from 70.7 to 93.5% (Komorowska et al. 2011). However, the criteria demarcating species in the genus *Foveavirus* is less than about 72% nt identity (or 80% aa identity) between their CP or polymerase genes (Adams et al. 2012). Comparison analysis of complete genome sequences of ASPV in the present work indicated that the sequence of ASPV 40/87 shared overall identities of 71.2% to 79.4% (section 3.4.2) with the other 12 isolates published in NCBI. It is concluded that ASPV40/87 is a diverged strain of ASPV infecting apple. Comparison analysis of the complete genome indicated that ASPV 40/87 shared a sequence identity of 78.7% with ASPV PB66, another strain kept in the virus collection of JKI, Dossenheim. An infectious cDNA clone of ASPV PB66 was constructed using CPEC method (Arntjen and Jelkmann 2009).

4.4 Development of ACLSV infectious cDNA clones as expression vector for foreign genes

Systemic infection of *N. occidentalis* 37B was successful with constructs pIF13-9 and pG11-15. In the construct of pIF13-9, a maker gene of EmGFP was fused to the 3' end of MP gene and the overlapped ORFs between CP and MP genes were duplicated (see section 2.2.2.3.1 and section 3.5.1). In the construct of pG11-15, a marker gene of mCherry was fused to the 5' end of CP gene, and the overlapped ORFs between CP and MP genes were also duplicated (see section 2.2.2.3.1 and section 3.5.1). The result of RT-PCR detection of marker genes in ACLSV in symptomatic *N. occidentialis* 37B suggested deletion of inserted EmGFP and mCherry (see section 3.5.3). Although there was no direct evidence, the deletion of marker genes can be a result from homologous recombination of the duplicated fragments of the overlapping ORFs. A similar phenomenon has been observed for TMV. Dawson et al. (1989) attempted to build a TMV-based vector using add-a-gene strategy. In one trial, the ORF of chloramphenicol acetyltransferase (CAT) under control by a subgenomic promoter of TMV was inserted before the coat protein gene (CAT-CP). The constructed vector was too unstable

for the CAT gene which was surrounded by the repeated sequences of the coat protein promoter (around 250 bp), and homologous recombination between those sequences resulted in deletion of the inserted sequences. In plasmids of pIF13-9 and pG11-15, the marker genes were inserted into duplicated fragments of the possible promoter of CP of ACLSV (300 bp). Therefore, it was assumed that the deletion of the marker genes (see section 3.5.3) could be due to the homologous recombination of the duplicated fragments.

The deletion of marker genes via homologous recombination can be reduced by increasing the heterogeneity of sequences. For example, recombination - deletion between homologous cassettes in retrovirus can be efficiently suppressed via a strategy of degenerate codon substitution in the two-chain single-vector (Im et al. 2014). In addition, it is reported that recombination frequency to DNA divergence fitted a log-linear function until approximately 10% sequence divergence in *Agrobacteria* (Costechareyre et al. 2009). To avoid the deletion of marker genes in pIF13-9 and pG11-15, which may cause by the homologous recombination of duplicated fragments, the plasmids of pIF18-2, pIF25-7, pIF24-6 and pIF23-1 were constructed. In these plasmids, the genes of ACLSV 38/85A (from the clone pIF3-15) and ACLSV 38/85B (from the clone pIF3-19) were recombined (see section 2.2.2.3). The results of RT-PCR detection of the marker genes (see section 3.5.3 and 3.5.4) indicated that the deletion of marker genes in the plasmids of pIF18-2, pIF24-6 and pIF23-1 has been avoided. However, no infection of *N. occidentalis* 37B was observed for any construct.

The expression of marker genes at 3' terminus of CP was not successful. It is known that enkephalin can be produced in tobacco protoplasts using TMV vectors, and the enkephalin is fused to the C-terminus of CP of TMV (Takamatsu et al. 1990). Moreover, CAT can be expressed when fused to C-terminus of CP of TMV, although CP-CAT moved poorly (Dawson et al. 1989). In the plasmids of pIF16-1 the marker gene of mCherry was inserted between CP gene (without stop codon) and 3' UTR of ACLSV. However, the results of western blot (see section 3.5.3) indicated that no viral protein or fluorescent protein was expressed from the three constructed plasmids. Given the possibilities that the function of CP was inhibited by the mCherry protein or the size of mCherry protein is too large to be a label for CP, the two other plasmids of pIF20-16 and pIF27-10 were constructed. In pIF20-16, a rigid linker of the alpha helix-forming linkers of $(EAAAK)_4$ was inserted between CP gene and mCherry gene of pIF16-1 to avoid interaction between the two proteins. Rigid linkers can serve to keep a fixed distance between functional domains, and therefore maintain their independent functions (Arai et al. 2001; Chen et al. 2013). In pIF27-10, a marker gene of

smaller size of 342 bp than mCherry (711 bp) was used instead of the mCherry in pIF16-1. However still there was no infection or viral protein expression was observed in the agroinoculated plants. Therefore the C-terminus of ACLSV CP may not be available for expression of foreign genes.

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Supplementary

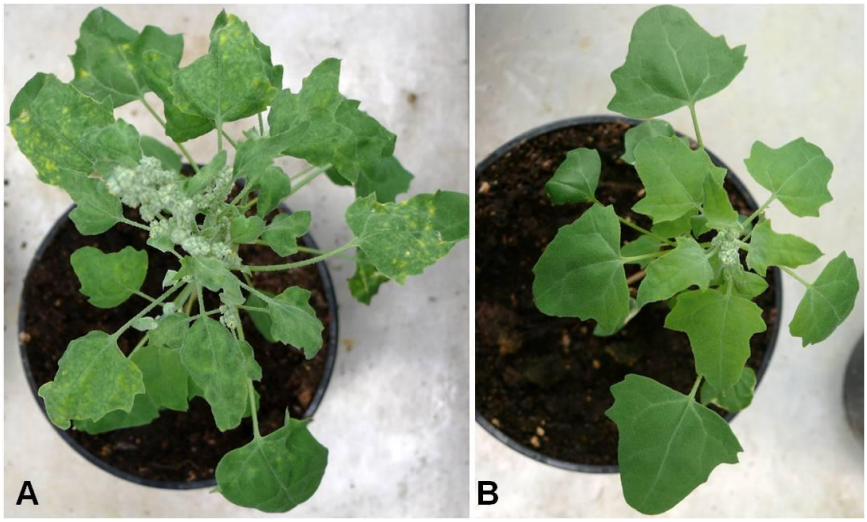

Figure S1 Systemic symptoms on *Chenopodium quinoa* infected with ACLSV by sap inoculation. A: an infected plant showing systemic symptoms of chlorosis and necrotic spots. B: a plant inoculated with sap of healthy N. occidentalis 37B, as a negative control.

Table S1 Primers used for sequencing the obtained ACLSV and ASPV cDNA clones

No.	Target clones	Forward primers	Sequences	Reverse primers	Sequences
1	pIF3-14	seq-f-03	TATCTTTCGCCTTTTGCTTACG	seq-r-03	GAAGTCGAACATAAGCTCTGGATA
1	pIF3-15	seq-f-04	AACTTCTCTTTCTATCTACCAG	seq-r-04	GACCTTTTGTTGGGCCTTATTCAT
1	pIF3-19	seq-f-05/03	TATCTTTCGCCTTTTGCTTACG	seq-r-05/03	GAAGTCGAACATAAGCTCTGGATA
1	pIF4-4	seq-f-05	CCTTCTGGTGATGTTTTTC	seq-r-05	GAGTTGGATGTGACAGTTCTTG
1	pIF14-23	seq-f-27	GGACCGTACGACCTGTTTGATGT	seq-r-27	GTCCCCTGCACCGCTATG
2	pIF3-14	seq-f-08	TCGTTGAAAAAGCCTGATGTT	seq-r-08	GCTTGGGTCGGAACTGTCTGG
2	pIF3-15	seq-f-09	AGGGCCCCGATTCAAGATT	seq-r-09	TTCCAAGCATCCCAAAAAG
2	pIF3-19	seq-f-10/08	TCGTTGAAAAAGCCTGATGTT	seq-r-10/08	GCTTGGGTCGGAACTGTCTGG
2	pIF4-4	seq-f-10	GCAGCAAACTTGAGAGCAT	seq-r-10	TGTGCAAGCAGGAGCGTTAG

No.	Target clones	Forward primers	Sequences	Reverse primers	Sequences
2	pIF14-23	seq-f-28	TTCCAAAAACCCCTTATG	seq-r-28	TTATTGAATCAGAAAGACC
3	pIF3-14	seq-f-13	CAAAGTTGAAGGAAAAAGGCAGAG	seq-r-13	ACCTTGTTAGCACTTGATGA
3	pIF3-15	seq-f-14	CATCAAGAAAGCAAAAGTCAAGTC	seq-r-14	TCCTCGTCAGATAACCACTCAAG
3	pIF3-19	seq-f-15/13	CAAAGTTGAAGGAAAAAGGCAGAG	seq-r-15/13	ACCTTGTTAGCACTTGATGA
3	pIF4-4	seq-f-15	GACTGTCAACTACTCTGGGGAAGC	seq-r-15	CTGACCCTGCCTGGCTTTCTTG
3	pIF14-23	seq-f-29	CATATCCGACATTTGAAGC	seq-r-29	GAATAAAGCCTCTCACCT
4	pIF3-14	seq-f-18	TGAGGGTACACAGAAGAGA	seq-r-18	TGCCCTGCTTTTGCTTCTGT
4	pIF3-15	seq-f-19	CTTGTGGGGAGGGAGAA	seq-r-19	ACCAAGGGCTGAACTCCACAAG
4	pIF3-19	seq-f-20/18	TGAGGGTACACAGAAGAGA	seq-r-20/18	TGCCCTGCTTTTGCTTCTGT
4	pIF4-4	seq-f-20	TGAAGCCAAAAATGACG	seq-r-20	GCCATAGTATTAAACAAGAAGGTG
4	pIF14-23	seq-f-30	TCAGAAGATCATCCATAGAC	seq-r-30	ATGTAGTAATTGTCAGGAAGAT
5	pIF3-14	seq-f-23	GGTCATATGCCCCAGAAG	seq-r-23	ATCGACCTCATGTAATCCT
5	pIF3-15	seq-f-24	GCATCAGTAAGGCGTTCATCC	seq-r-24	GCTCCAATTCCTGAACACATCCTC
5	pIF3-19	seq-f-25/23	GGTCATATGCCCCAGAAG	seq-r-25/23	ATCGACCTCATGTAATCCT
5	pIF4-4	seq-f-25	AAGTGCCCTAGATTGCTCA	seq-r-25	CTCAGCAGCATTCGTCA

Publication

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