Optimization and implementation of a bimolecular fluorescence complementation (BiFC) system for the detection of plum pox potyviral protein-protein interactions *in planta*

Von der Naturwissenschaftlichen Fakultät
der Gottfried Wilhelm Leibniz Universität Hannover
zur Erlangung des Grades

DOKTORIN DER NATURWISSENSCHAFTEN
Dr. rer. nat.

genehmigte Dissertation
von

Dipl.-Biol. Eva Zilian
geboren am 5. Januar 1981 in Gelsenkirchen

2011

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Tag der Promotion: 8. September 2011

ABSTRACT

Plum pox virus (PPV) belongs to the genus *Potyvirus* within the family *Potyviridae*. PPV and other members of the genus like *Turnip mosaic virus* (TuMV) or *Soybean mosaic virus* (SMV) are of agri- and horticultural importance causing significant losses in a wide range of crop plants. The potyviral genome consists of a single-stranded positive-sense RNA molecule, which encodes a polyprotein precursor that is processed by virus encoded proteinases to release ten viral proteins. An eleventh protein, which is encoded in a short open reading frame (ORF) embedded within the known ORF has been identified recently. Complete interaction maps for the ten proteins of several potyviruses have already been developed employing yeast two-hybrid (YTH) systems. However, limited information is available about interactions *in planta*. Consequently, the scope of the thesis was to develop an interaction map for the first ten PPV proteins *in planta.*

For this purpose, a bimolecular fluorescence complementation (BiFC) system, which enables detection of protein-protein interactions *in planta,* was optimized in the first part of this work. BiFC is based on the expression of two proteins of interest fused to the non-fluorescent N-terminal and C-terminal fragment, respectively, of a fluorescent protein. When both fragments are brought into tight contact by interaction of the two fused proteins a functional fluorescent protein is reconstituted. For the experiments in this work, a BiFC system, which is based on a monomeric red fluorescent protein (mRFP) was chosen. The expression plasmids, one encoding the N-terminal amino acids (aa) 1-168 and the other one encoding the C-terminal aa 169-225 of the mRFP, were modified in different steps. Firstly, a seven aa encoding linker sequence was integrated between the mRFP sequences and the genes of interest to allow maximal flexibility of the fused protein fragments for complex formation. Secondly, a modified mini binary plasmid replaced the original binary plasmid due to easier handling and higher stability. Furthermore, two different sets of plasmids were developed, which encode N- or C-terminal fusions of a split mRFP with the proteins of interest. For interaction studies the plasmids were electroporated in *Agrobacterium tumefaciens*, single *Nicotiana benthamiana* leaves were inoculated with a mixture of these agrobacteria and fluorescence was monitored three days past inoculation by confocal laser scanning microscopy. To enable an investigation of protein-protein interactions,

interacting and non-interacting controls were developed, consisting of the coat protein (CP) of PPV and the C-terminal aa 222-315 of CP, respectively. Finally, the functionality of the system was validated with proteins of other viruses from different genera, which were the N-protein of *Capsicum chlorosis virus*, the CP of *Tobacco mosaic virus* and the BC1 and BV1 of *Tomato yellow leaf curl Thailand virus*.

In the second part of this work the PPV protein interaction map was developed. Therefore, the ten PPV proteins P1, HC-Pro, P3, 6K1, CI, 6K2, VPg, NIa-Pro, NIb and CP were tested in all combinations, fused to both the mRFPN and the mRFPC fragment, for interaction studies. 52 of 100 possible interactions were detected, including the self-interactions of CI, 6K2, VPg, NIa-Pro and CP, representing more interactions than ever detected for any other potyvirus in a YTH approach.

Additionally, the BiFC system was found to be useful for the detection of subcellular localization of the self-interacting proteins. Furthermore, experiments with the eleventh PPV protein, P3N-PIPO, which is not well characterized so far, were conducted. P3N-PIPO was found to interact with CI, but neither with itself nor with CP. The P3N-PIPO-CI interaction was found to occur probably at plasmodesmata, which is in accordance to previous studies with P3N-PIPO and CI of TuMV supporting the hypothesis of an involvement of P3N-PIPO in the cell-to-cell movement of potyviruses.

For HC-Pro only one interaction with CI was detected. However, at least one further interaction, in particular self-interaction of the protein, was expected, but was not identified *in planta.* To verify this result, further experiments with HC-Pro of PPV and TuMV, for which a self-interaction *in planta* has been shown recently, were conducted. A self-interaction of TuMV_HC-Pro was demonstrated. Furthermore, two mutants of both HC-Pros were generated and self-interaction of all mutants was detected in the BiFC. These results indicate that HC-Pro self-interaction might be species-specific.

The study delivers a reliable system for the detection of protein-protein interactions and presents the first interaction map for a potyvirus developed *in planta*. The obtained results could contribute to a better understanding of the interplay of proteins during the virus infection cycle.

Keywords: *Plum pox virus* (PPV), protein-protein interactions, bimolecular fluorescence complementation (BiFC)

ZUSAMMENFASSUNG

Das *Plum pox virus* (PPV) gehört zum Genus *Potyvirus* innerhalb der Familie *Potyviridae.* Das Virus und andere Mitglieder des Genus, z.B. das *Turnip mosaic virus* (TuMV) oder das *Soybean mosaic virus* (SMV) sind von großer Bedeutung für Landwirtschaft und Gartenbau, da sie erhebliche Verluste bei vielen Kulturpflanzen verursachen. Das Genom der Potyviren besteht aus einem einzelsträngigen RNA Molekül, das ein Polyprotein kodiert, welches durch viruseigene Proteinasen in zehn virale Proteine prozessiert wird. Erst vor Kurzem wurde ein elftes Protein identifiziert, das auf einem kurzen offenen Leseraster (ORF) innerhalb des bekannten ORFs kodiert wird. Für die ersten zehn Proteine verschiedener Potyviren wurden bereits komplette Interaktionskarten mittels 'Hefe-Two-Hybrid'-Systemen erstellt. Jedoch sind nur wenige Informationen über Interaktionen *in planta* verfügbar. Daher war das Ziel dieser Arbeit, für die ersten zehn PPV Proteine eine komplette Interaktionskarte *in planta* zu erstellen.

Zu diesem Zweck wurde im ersten Teil dieser Arbeit ein bimolekulares Fluoreszenzkomplementationssystem (BiFC), das die Detektion von Protein-Protein Interaktionen *in planta* ermöglicht, optimiert. BiFC basiert auf der Expression zweier Zielproteine, die an das nicht fluoreszierende N-terminale Fragment bzw. C-terminale Fragment eines Fluoreszenzproteins fusioniert sind. Interagieren diese beiden Proteine miteinander, so werden die Fragmente des Fluoreszenzproteins in räumliche Nähe zueinander gebracht und rekonstituieren zu einem funktionellen Protein. In dieser Arbeit wurde ein BiFC-System verwendet, das auf einem monomeren rot fluoreszierenden Protein (mRFP) beruht. Die Expressionsplasmide, die die N-terminalen Aminosäuren (AS) 1-168 bzw. die C-terminalen AS 169-225 des mRFP kodieren, konnten in verschiedenen Schritten modifiziert werden. Zunächst wurde eine Sequenz, die einen sieben AS langen Linker kodiert, zwischen die mRFP-Sequenzen und die für die Proteine kodierenden Bereiche integriert. Dieser Linker sollte maximale Flexibilität der beiden Fusionsteile bei der Komplexbildung gewährleisten. Als zweites wurde das binäre Originalplasmid durch ein modifiziertes binäres Miniplasmid ersetzt, das eine einfachere Handhabung und höhere Stabilität der Konstrukte sicherstellen sollte. Des Weiteren ermöglichte die Erstellung zweier Plasmidsets die Expression sowohl N-terminaler als auch C-terminaler mRFP-Fusionen mit den Zielproteinen. Die Plasmide

wurden in *Agrobacterium tumefaciens* elektroporiert, Blätter von *Nicotiana benthamiana* Pflanzen wurden mit Mischungen dieser Agrobakterien inokuliert und Fluoreszenz konnte drei Tage nach der Inokulation mittels konfokaler Laserscan-mikroskopie überprüft werden. Zudem wurden eine interagierende und eine nicht-interagierende Kontrolle entwickelt, bestehend aus dem Hüllprotein (CP) des PPV bzw. dem C-terminale Bereich (AS 222-315) dieses Proteins. Schließlich konnte die Funktionalität des Systems mit Proteinen von Viren unterschiedlicher Genera, dem N-Protein des *Capsicum chlorosis virus*, dem CP des *Tobacco mosaic virus* und den Proteinen BV1 und BC1 des *Tomato yellow leaf curl Thailand virus,* nochmals bestätigt werden.

Im zweiten Teil der Arbeit wurde die Interaktionskarte für die Proteine des PPV erstellt. Die zehn PPV Proteine P1, HC-Pro, P3, 6K1, CI, 6K2, VPg, NIa-Pro, NIb und CP wurden dazu in allen Kombinationen, sowohl als Fusion mit dem mRFPN- als auch mit dem mRFPC-Fragment auf mögliche Interaktionen getestet. Von 100 möglichen konnten 52 Interaktionen identifiziert werden, darunter die Selbstinteraktionen der Proteine CI, 6K2, VPg, NIa-Pro und CP. Dies sind mehr Interaktionen als jemals zuvor für ein Potyvirus mit einem ‘Hefe-Two-Hybrid’-System gefunden wurden. Weiterhin konnte gezeigt werden, dass das BiFC-System die Lokalisierung von Protein-Protein Interaktionen auf subzellulärer Ebene ermöglicht, was beispielhaft anhand der genannten Selbstinteraktionen demonstriert wurde. Zudem wurden weitere Versuche mit dem elften PPV Protein, P3N-PIPO, das bisher noch nicht gut charakterisiert ist, durchgeführt. Es konnte eine Interaktion zwischen P3N-PIPO und CI, aber keine Selbstinteraktion oder Interaktion mit dem CP identifiziert werden. Die P3N-PIPO-CI Interaktion wurde in Strukturen lokalisiert, die möglicherweise Plasmodesmata darstellen. Dies ist in Übereinstimmung mit Ergebnissen aus Studien mit P3N-PIPO und CI des TuMV und stützt die Hypothese, dass P3N-PIPO am Zell-zu-Zell Transport der Potyviren beteiligt sein könnte.

Für HC-Pro konnte in dieser Arbeit lediglich eine Interaktion mit CI identifiziert werden. Zumindest Selbstinteraktion des Proteins wurde erwartet, konnte aber nicht *in planta* gezeigt werden. Um dieses Ergebnisse nochmals abzusichern, wurden weitere Experimente mit dem HC-Pro des PPV und des TuMV durchgeführt. Für letzteres wurde erst kürzlich eine Selbstinteraktion *in planta* demonstriert, die in dieser Arbeit bestätigt werden konnte. Weiterhin wurden von beiden HC-Pros zwei Mutanten erzeugt,

für die alle mittels BiFC eine Selbstinteraktion gezeigt werden konnte. Die Ergebnisse lassen vermuten, dass die Selbstinteraktion des HC-Pros spezies-abhängig sein könnte.

Diese Arbeit liefert ein verlässliches System zur Detektion von Protein-Protein-Interaktionen und zeigt die erste Interaktionskarte für Proteine eines Potyvirus, die *in planta* entwickelt wurde. Zudem tragen die Ergebnisse zu einem besseren Verständnis des Zusammenspiels von Proteinen während des viralen Infektionszyklus bei.

Schlagworte: *Plum pox virus* (PPV), Protein-Protein Interaktionen, Bimolekulare Fluoreszenzkomplementation (BiFC)

CONTENTS

ABBREVIATIONS

%	Percentage
∅	Diameter
~	Approximately
°C	Degree Celsius
6K1	First protein of 6 kilodalton
6K2	Second protein of 6 kilodalton
35SpA	Cauliflower mosaic virus 35S polyadenylation signal
A	Adenine (nucleobase)
A	Alanine (amino acid)
aa	Amino acid(s)
AD	Activation domain
AP-1	Activator protein 1
AT	Aphid-transmissible
A. thaliana	*Arabidopsis thaliana*
attR2	Attachment-site 2 of the Gateway® -Cloning system
A. tumefaciens C58C1	*Agrobacterium tumefaciens* strain C58C1
B42	Transcriptional activation domain of an *Escherichia coli* protein
BC1	Movement protein of begomoviruses
BFP	Blue fluorescent protein
BHK	Baby hamster kidney cells
BiFC	Bimolecular fluorescence complementation
BIND	'Biomolecular Interaction Network Database'
bp	Base pairs
BRET	Bioluminescence resonance energy transfer
BV1	Nuclear shuttle protein of begomoviruses
bZIP	Basic leucine zipper
C	Cysteine (amino acid)
C	Cytosine (nucleobase)
CFP	Cyan fluorescent protein
CI	Cylindrical inclusion protein
CLSM	Confocal laser scanning microscopy

cm	Centimeter
c-myc	C-terminal region of the human c-Myc protein
Co-IP	Co-Immunoprecipitation
CP	Coat protein
CP_{full}	Full-length coat protein
C-terminus /-terminal	Carboxyl terminus /-terminal
D	Aspartic acid (amino acid)
DBD	DNA-binding domain
DIP	'Database of Interacting Proteins'
DNA	Desoxyribonucleic acid
DNase	Desoxyribonulease
dpi	Days past inoculation
DSMZ	'Deutsche Sammlung für Mikroorganismen und Zellkulturen'
DsRed	Red fluorescent protein from the coral *Discosoma striata*
E	Glutamic acid (amono acid)
E. coli	*Escherichia coli*
e.g.	Exempli gratia (Latin: for example)
ER	Endoplasmatic reticulum
et al.	Et alii (Latin: and others)
F	Phenylalanine (amino acid)
Fig.	Figure
FRET	Fluorescence resonance energy transfer
G	Glycin (amino acid)
G	Guanine (nucleobase)
GAL4	Yeast GAL4 transcription activator
GFP	Green fluorescent protein
GOI	Gene(s) of interest
GreNe	Green neon
GST	Glutathion-S-transferase
GUS	*Escherichia coli* β-Glucuronidase
H_2O_2	Hydrogen peroxide
H	Histidine (amino acid)
h	Hour(s)

HA	Hemagglutinin-tag
HC-Pro	Helpercomponent-proteinase
HRP	Horseradish peroxidase
I	Isoleucine (amino acid)
IgG	Immunoglobulin G
K	Lysine (amino acid)
kb	Kilobase pairs / kilobases
KCl	Potassium chloride
kDa	Kilodaltons
KH_2PO_4	Potassium dihydrogen phosphate
L	Leucine (amino acid)
LexA	Bacterial LexA repressor protein
M	Marker
mCherry	Monomeric Cherry protein (a red fluorescent protein)
MCS	Multiple cloning site
MES	2-[N-morpholino]ethanesulfonic acid
$MgCl_2$	Magnesium chloride
min	Minute(s)
ml	Milliliter(s)
mLumin	Monomeric far-red fluorescent protein
mM	Millimolar
MP	Movement protein
mRFP	Monomeric red fluorescent protein
mRFPC	C-terminal fragment of the monomeric red fluorescent protein
mRFPN	N-terminal fragment of the monomeric red fluorescent protein
µm	Micrometer(s)
NaCl	Sodium chloride
Na_2HPO_4	Di-sodium hydrogen phosphate dihydrate
NAT	Non-aphid-transmissible
N. benthamiana	*Nicotiana benthamiana*
NES	Nuclear export signal
NIa-Pro	Nuclear inclusion protein a-proteinase
NIb	Nuclear inclusion protein b

NLS	Nuclear localization signal
nm	Nanometer(s)
N-protein	Nucleocapsid protein of tospoviruses
N-terminus /-terminal	Amino-terminus /-terminal
NTPase	Nucleosidtriphosphatase
NTR	Non-translated regions
OD_{600}	Optical density measured at 600 nm
ORF	Open reading frame
P	Proline (amino acid)
P1	Protein 1
P3	Protein 3
p19	P19 - suppressor of gene silencing of *Tomato bushy stunt virus*
P3N-PIPO	Protein fusion of the N-terminus of P3 and PIPO
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
pe35S	Enhanced Cauliflower mosaic virus 35S promoter
pH	Negative decade logarithm of hydrogen ion concentration
PIPO	Pretty Interesting *Potyviridae* ORF
poly-A	Polyadenylation signal
Q	Glutamine (amino acid)
R	Arginine (amino acid)
RBS	Ribosomal binding site
RdRp	RNA-dependent RNA polymerase
RFP	Red fluorescent protein
RNA	Ribonucleic acid
RT	Reverse transcription
S	Serine (amino acid)
SDS	Sodium dodecyl sulfate
SEL	Size exclusion limit
Sos	‘Son of sevenless’ putative guanine nucleotide exchange factor
ssRNA	single-stranded ribonucleic acid
T	Threonine (amino acid)

T	Thymine (nucleobase)
Tab.	Table
TAP	Tandem affinity purification
TL	Translational enhancer
UAS	Upstream activating sequence
V	Valine (amino acid)
VPg	Viral genome linked protein
Y	Tyrosine (amino acid)
YFP	Yellow fluorescent protein
YTH	Yeast two-hybrid

Virus acronyms

AbMV	*Abutilon mosaic virus*
CaCV	*Capsicum chlorosis virus*
CaMV	*Cauliflower mosaic virus*
JGMV	*Johnsongrass mosaic virus*
LMV	*Lettuce mosaic virus*
PPV	*Plum pox virus*
PRSV	*Papaya ringspot virus*
PSbMV	*Pea seed-borne mosaic virus*
PVA	*Potato virus A*
PVY	*Potato virus Y*
PYDV	*Potato yellow dwarf virus*
SMV	*Soybean mosaic virus*
SYSV	*Shallot yellow stripe virus*
TBSV	*Tomato bushy stunt virus*
TEV	*Tobacco etch virus*
TMV	*Tobacco mosaic virus*
TuMV	*Turnip mosaic virus*
TVMV	*Tobacco vein mottling virus*
TYLCTHV	*Tomato yellow leaf curl Thailand virus*

1 General introduction

1.1 Plant viruses of the genus *Potyvirus*

The genus *Potyvirus* is the major genus within the family *Potyviridae* and constitute the largest group of plant-infecting viruses including approximately 30% of all known plant viruses (Ward & Shukla, 1991; Shukla *et al.*, 1994). Members of the genus are of agricultural and horticultural importance, because they infect a broad range of host plants, both monocotyledonous and dicotyledonous, from several families in many climatic regions. As a consequence they are responsible for significant agricultural and economic damage (Ward & Shukla, 1991). Symptoms of potyvirus-infected plants are very diverse, depending on the host plant and the virus strain and can be observed in all parts of a plant including fruits, tubers or bulbs. As representatives of the genus four different viruses are introduced to demonstrate the diversity among potyviruses.

Firstly, *Potato virus Y* (PVY), the type member of the genus, is one of the most important potato viruses world-wide by significantly decreasing the yield. It infects preferably plants of the family *Solanaceae,* e.g., potato, tomato, pepper and tobacco plants, but also plants in the *Leguminaceae* and *Chenopodiaceae* are hosts of PVY (de Bokx & Huttinga, 1981). As well as PVY, the *Turnip mosaic virus* (TuMV) occurs throughout the world. It is known to infect around 318 species in over 43 families, among them primarily *Brassicaceae, Cruciferae* and *Chenopodiaceae,* which represent the broadest known host range of any potyvirus (Walsh & Jenner, 2002; Ohshima, 2008). A further important representative of the genus is the *Soybean mosaic virus* (SMV). As it is present in all major soybean-growing areas it is one of the most economically important viruses of soybean (Cho & Goodman, 1979). Finally, the fourth important virus and in focus of this thesis is the *Plum pox virus* (PPV), which causes the sharka disease, first described on plums in Bulgaria (Atanasoff, 1932). It occurs world-wide and is described as the most devastating virus disease on wild and cultivated *Prunus* species. Especially in plum, apricot and peach trees, the disease is very detrimental by reducing quality and inducing premature dropping of fruits, which implies a decline in yield (Dunez & Sutic, 1988; Németh, 1994; Cambra *et al.*, 2006). Symptoms upon infection reach from chlorotic diffuse or ring-shaped spots, leaf vein chlorosis or deformation of infected leaves to diverse symptoms on fruits. The fruits of

infected plants are malformed, contain light rings on the skin and stones and have a lower sugar content (Kegler & Schade, 1971; Subr & Glasa, 2008; Fig. 1a-d).

In general, there are different means of plant-to-plant spread of plant viruses. Most plant viruses are depending for transmission on another organism, called a vector. Typical vectors are aphids, whiteflies, thrips, mites, but also further organisms are able to transmit plant viruses (Andret-Link & Fuchs, 2005). Potyviruses are predominantly transmitted by a number of aphid species (Fig. 1e-f) in a non-persistent manner with only short times of acquisition and retention (Kegler & Schade, 1971; Labonne *et al.*, 1995; Ng & Falk, 2006). Furthermore, most potyviruses including PPV may also be transmitted mechanically (Kegler & Schade, 1971), that means, e.g., introduced through a wound into the plant or by grafting. And finally, there are even some seed-transmissible potyviruses (Mink, 1993; Johansen *et al.*, 1994; Shukla *et al.*, 1994), like SMV (Porto & Hagedorn, 1975). For several potyviruses additional non-aphid-transmissible (NAT) isolates have been described (Maiss *et al.*, 1989; Nakashima *et al.*, 1991; Lopez-Moya *et al.*, 1995; Andrejeva *et al.*, 1996). The mechanism of aphid-transmission of potyviruses as well as reasons for the occurrence of NAT-strains have been studied intensively over the last decades. This lead to the development of a model for the process of transmission involving two potyviral proteins, the coat protein (CP) and the helpercomponent-proteinase (HC-Pro), which is described in chapter 1.1.2.

Fig. 1: Images of different plants and fruits infected with *Plum pox virus* (PPV) (a-d) and two representative vectors of PPV (e-f). PPV symptoms on leaves (http://pflanzengesundheit.jki.bund.de/index.php?menuid=60&reporeid=244) (a) and fruits (http://www.forestryimages.org/ browse/ detail.cfm?imgnum=0660082) of *Prunus domestica* (b), and on leaves (c) and fruits of *P. persicae* (www.agf.gov.bc.ca/cropprot/ppv.htm) (d). Pictures of the green peach aphid *Myzus persicae* (www.ipmimages.org/browse/detail.cfm?imgnum=1317037) (e) and the cowpea aphid *Aphid craccivora* (http://bugguide.net/node/view/356327/bgpage) (f).

1.1.1 Particle structure and genome organization

Potyviral virions are flexible filaments with a length of 680-900 nm and a width of 11-15 nm (Fig. 2). They consist of a non-enveloped capsid, which is made up of about 2000 subunits of a single type of structural protein, the CP. The capsid is elongated with helical symmetry and encapsulates the potyviral genome - a single-stranded, positive-sense RNA molecule with a length of approximately 10 kilobase pairs (kb) (Dougherty & Carrington, 1988; Riechmann *et al.*, 1992).

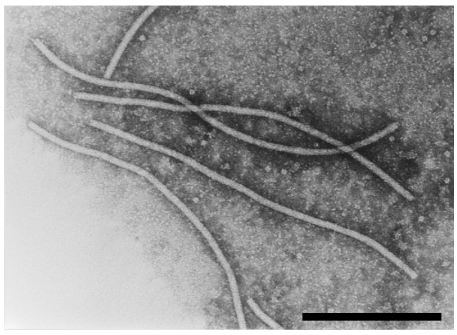

Fig. 2: Image of potyviral particles. Electron micrograph of purified *Potato virus Y* (PVY) particles, representative for members of the genus *Potyvirus*. Scale bar: 350 µm (Brunt *et al.*, 1996).

At its 5`-end the potyviral RNA is covalently linked to a viral protein, the VPg (viral genome linked protein; Hari, 1981; Riechmann *et al.*, 1989) and the 3`-end carries a poly-A-tail (Hari *et al.*, 1979). At both the 5`- and the 3`-end there are non-translated regions (5`-NTR and 3`-NTR). The RNA comprises one long open reading frame (ORF), which is translated into a polyprotein precursor of circa 350 kilodaltons (kDa). This precursor is post-translational processed by three virus-encoded proteinases to yield ten mature proteins (Fig. 3) and these are listed from the 5`-end to the 3`-end: the protein 1 (P1), the HC-Pro, the protein 3 (P3), a first peptide of 6 kDa (6K1), the cylindrical inclusion protein (CI), a second peptide of 6 kDa (6K2), the nuclear inclusion protein a (NIa) with the amino-terminal (N-terminal) VPg and a carboxyl-terminal (C-terminal) proteinase (NIa-Pro), the nuclear inclusion protein b (NIb) and the CP (Riechmann *et al.*, 1992; Shukla *et al.*, 1994; López-Moya *et al.*, 2000).

Recently the discovery of a further short ORF, called PIPO (pretty interesting *Potyviridae* ORF), was reported for TuMV (Chung *et al.*, 2008; Wei *et al.*, 2010b) and other potyviruses (Wen & Hajimorad, 2010). This short ORF is embedded within the P3 cistron. In consequence, an additional 6-7 kDa protein is produced from the P3 protein-coding region by frameshifting into a +2 reading frame (Chung *et al.*, 2008) as a protein

fusion with the N-terminal part of P3, resulting in a fusion protein of approximately 25 kDa (P3N-PIPO).

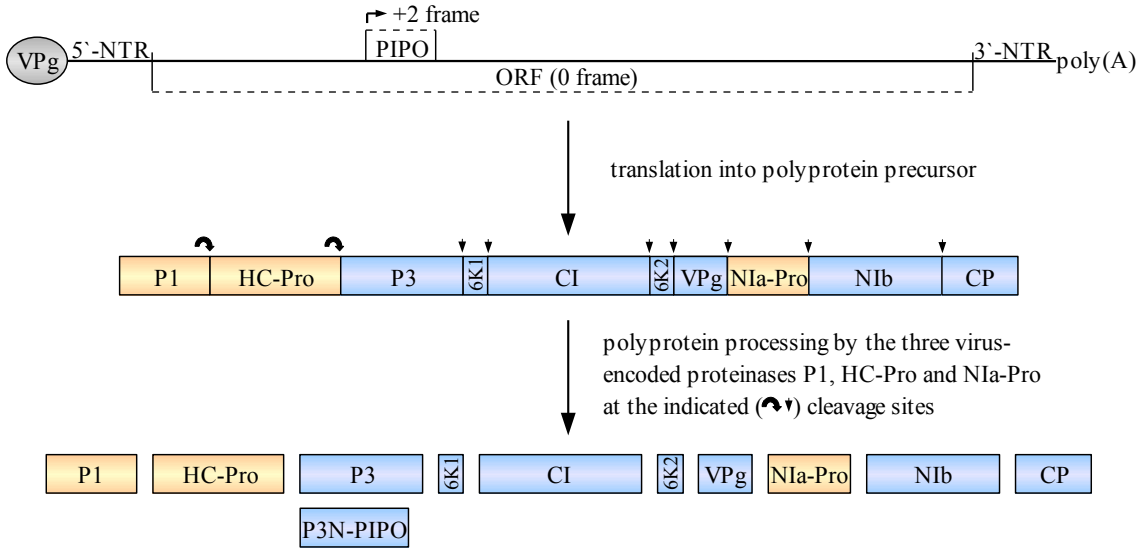

Fig. 3: Schematic representation of the potyviral genome organization and the polyprotein-processing. The RNA sequence is indicated as a black line, and the long ORF and PIPO are presented with dashed lines. VPg and 5`-NTR at the 5`-end and 3`-NTR and poly(A)-tail at the 3`-end are indicated. The potyviral polyprotein is represented by a box and the positions of the three proteinases are highlighted in orange. Cleavage sites for the proteinases are indicated with arrowheads, whereby the two rounded arrowheads represent the sites for autocatalytical cleavage of P1 and HC-Pro and further arrowheads indicate the cleavage sites of the NIa-Pro. In the lower part of the scheme the mature proteins, including P3N-PIPO fusion protein, are displayed. For abbreviations see text.

The polyprotein processing is mediated by the viral proteinases P1, HC-Pro and NIa-Pro, whereby the P1 and the HC-Pro catalyze the cleavage at their respective C-termini and NIa-Pro mediates the further cleavage reactions (Carrington *et al.*, 1989; Verchot *et al.*, 1991; Merits *et al.*, 2002). This proteolytic cleavage occurs at conserved amino acid (aa) motifs within the polyprotein (Adams *et al.*, 2005a), which are denoted in Fig. 3 with short arrowheads. Thereby, different cleavage sites are processed with different efficiencies, resulting in the occurrence of different additional intermediates (Merits *et al.*, 2002), not presented in the scheme.

1.1.2 Replication, movement and transmission

After infection, the virus genome is replicated. This replication process of potyviral RNA genomes, also called genome amplification, takes place in the cytoplasm of plant cells and involves a number of potyviral proteins as well as different host factors. The basic mechanism of the replication process of positive-sense RNA viruses comprise

firstly, that in a replication complex a complementary RNA strand is synthesized using the positive-strand RNA as template and secondly, that the newly synthesized negative-strand is in turn used as template for the generation of new positive-stranded RNA genomes (Hull, 2002).

Although most potyviral proteins are supposed to function at different steps in genome replication, the replication complex is believed to contain the following proteins: the NIb, as the key protein by acting as RNA-dependent RNA polymerase (RdRp; Hong & Hunt, 1996), the CI as helicase to unravel double-stranded RNA complexes, which are produced during replication (Laín *et al.*, 1990) and the NIa-Pro, which is believed to function through an interaction with NIb (Hong *et al.*, 1995) as stimulator for NIb polymerase activity (Fellers *et al.*, 1998). The 6K2 protein is a membrane-bound protein (Restrepo-Hartwig & Carrington, 1994) and is suggested to anchor the replication complex to the site of replication at the endoplasmatic reticulum (ER)-like membranes (Schaad *et al.*, 1997a). Since NIa-Pro has RNA-binding activity, it directs the viral RNA into the replication complex. VPg, which is covalently linked to the viral genomic RNA may act as primer for synthesis of the RNA during replication (Murphy *et al.*, 1996). NIb then might be recruited by the 6K2/VPg/NIa-Pro complex by an interaction with NIa-Pro to fulfill its function as polymerase. Furthermore, different host factors were supposed to be required for the recruitment and assembly of the complete replication complex (Ahlquist *et al*., 2003) and some were shown to interact with NIb (Wang *et al.*, 2000) or VPg (Wittmann *et al.*, 1997; Robaglia & Caranta, 2006). However, the exact mechanism of formation and proceeding of the potyviral replication complex has not yet been resolved.

After replication of the virus in single cells, the virus moves through the plant to establish infection also in distant regions of the plant (Carrington *et al.*, 1996). This firstly requires movement of the virus from cell-to-cell through plasmodesmata, representing microscopic channels, which pass the cell walls. Secondly, long-distance movement of the virus within the vascular system of the plant enables a spread throughout the plant (Revers *et al.*, 1999).

The cell-to-cell movement of potyviruses is, unlike of other viruses, not depending on a specialized movement protein (MP), but CP and HC-Pro were suggested to provide the classical functions of MPs (Dolja *et al.*, 1994; Rojas *et al.*, 1997). They are presumed to modify the size exclusion limit (SEL) of plasmodesmata and mediate the movement of

viral RNA from cell to cell (Rojas *et al.*, 1997). As a further protein the CI is involved in cell-to-cell-movement (Carrington *et al.*, 1998). The CI is known to form cylindrical inclusions in the cytoplasm of infected cells (Lawson & Hearon, 1971), and these inclusions were also found to form cone-shaped structures at or near plasmodesmata. These structures contain a continuous channel through the center of the CI proteins and the plasmodesmata (Rodríguez-Cerezo *et al.*, 1997; Roberts *et al.*, 1998) and direct intracellular translocation of the viral transport complex, which contains the CP (Carrington *et al.*, 1998; Roberts *et al.*, 1998). It is not understood so far whether potyviruses move from cell to cell as virions or as ribonucleoprotein-complexes, but a transport of complete virions is presumed. Moreover, recent studies identified P3N-PIPO as additional protein involved in cell-to-cell movement (Wei *et al.*, 2010b; Wen & Hajimorad, 2010) and an extended model for the viral transport through plasmodesmata was postulated by Wei *et al.* (2010). By this, P3N-PIPO modulates the localization of CI-virion complexes to the plasmodesmata and CI conical structures grow at the P3N-PIPO, which is anchored at the plasmodesmata. These CI structures then probably recruit further virus particles for the transfer through the plasmodesmata (Wei *et al.,* 2010b; Niehl & Heinlein, 2011).

By cell-to-cell movement the virus can reach the phloem sieve cells, which is followed by a passive translocation in the phloem to a distant site of the plant (Carrington *et al.*, 1996). So much research about the mechanism of potyviral cell-to-cell movement was conducted, so less is known about the long-distance movement and the roles of potyviral proteins involved in this process. At least three viral proteins were supposed to be involved, namely CP, HC-Pro and VPg, whereby CP and VPg are indicated to be included in a viral transport complex (Dolja *et al.*, 1994, 1995; Cronin *et al.*, 1995; Schaad *et al.*, 1997b; Revers *et al.*, 1999). However, due to the difficulty of analyzing this process, exact models for long-distance movement are still missing.

From infected plants viruses can be transmitted to other plants, as described above, predominantly by different aphids in a non-persistent manner (Labonne *et al.*, 1995; Ng & Falk, 2006). Aphids probe before they are feeding on sap of phloem vessels, thereby virus is incorporated into the aphid stylet. By tapping cells of uninfected plants the virus might be subsequently introduced into the healthy plant to establish an infection. The potyviral proteins CP and HC-Pro were identified to be involved in this process leading to the so called ‘bridging-model’. By this, firstly, the HC-Pro binds to

the food channel of the aphid stylet and secondly CP of the virions bind to the HC-Pro (Govier & Kassanis, 1974; Pirone & Blanc, 1996). For both proteins conserved aa domains that are directly involved in the binding have been characterized. In most cases, the aa triplet DAG in the N-terminal region of potyviral CP (Atreya *et al.*, 1991) and an N-terminal KITC-motif (Blanc *et al.*, 1998) as well as a C-terminal PTK-motif (Peng *et al.*, 1998) in the HC-Pro were shown to be essential for aphid transmission. The PTK-motif of the HC-Pro binds to the DAG of the potyviral CP, whereas the KITC-motif of HC-Pro is suggested to be involved in the interaction with the aphid stylet (Blanc *et al.*, 1998). Recently, an interaction between HC-Pro of *Tobacco etch virus* (TEV) and an aphid ribosomal protein was shown to be involved in the binding of HC-Pro to the aphid stylet (Fernández-Calvino *et al.*, 2010). Ng & Falk (2006) demonstrated that the interaction between HC-Pro and CP is not species-specific, but that CP of one potyvirus can enable aphid transmission by interaction with the HC-Pro of another potyvirus. Mutations in the mentioned motifs can prevent transmission and are the reason for the occurrence of NAT-strains as described in chapter 1.1.

1.1.3 The functions of potyviral proteins

Most of the potyviral proteins are multifunctional and several participate, as already partially mentioned above, in different phases of the virus infection cycle. An extensive overview on potyviral protein functions has been given previously by Urcuqui-Inchima *et al.* (2001) and by Rajamäki *et al.* (2004). The following section deals only with a short summary of the key functions of potyviral proteins to give an focused overview on the protein network.

P1 is the most divergent protein among all eleven potyviral proteins with regard to its length and aa sequence (Adams *et al.*, 2005b). Additionally to the already mentioned proteinase activity in the cleavage of the polyprotein precursor (Verchot *et al.*, 1991) P1 is suggested to be associated with symptomatology (Wisler *et al.*, 1995) and important in defining the potyvirus host range (Salvador *et al.*, 2008). Additionally, it might be involved in genome replication (Verchot & Carrington, 1995) and an accessory factor for HC-Pro in RNA-silencing suppression (Kasschau & Carrington, 1998; Valli *et al.*, 2006).

The functions of **HC-Pro** have been reviewed in several articles (Maia *et al.*, 1996; Syller, 2006). Its involvement in aphid-transmission and proteolytic activity in polyprotein processing have already been mentioned above. Furthermore, the protein increase SEL of plasmodesmata in cell-to-cell movement (Rojas *et al.*, 1997) and was shown to be involved in long-distance movement (Cronin *et al.*, 1995). Beyond that, HC-Pro acts as an effective suppressor of post-transcriptional gene silencing in plants (Voinnet *et al.*, 1999; Qu & Morris, 2005). Ala-Poikela *et al.* (2011) identified HC-Pro interaction with an eukaryotic initiation factor suggesting further roles in the virus infection cycle.

In comparison to all other potyviral proteins little is known about **P3** as well as **6K1**. P3 is suggested to be involved in virus replication (Merits *et al.*, 1999) and was identified as a pathogenicity determinant (Jenner *et al.*, 2003; Suehiro *et al.*, 2004). The functions of **6K1** are poorly understood. However, the deletion of 6K1 from the genome of *Potato virus A* (PVA) resulted in non-infectious mutants (Merits *et al.*, 2002) revealing the requirement of 6K1 for PVA infectivity in plants.

Beside its key-function in cell-to-cell movement (Gomez de Cédron *et al.*, 2006; Niehl & Heinlein, 2011) the **CI** plays a pivotal role in genome replication. The protein has nucleosidtriphosphatase (NTPase) and RNA helicase activities (Eagles *et al.* 1994; Laín *et al.* 1990, 1991), which have been shown to be essential for the genome replication (Fernández *et al.*, 1997).

6K2 was suggested to function in genome replication by anchoring the replication complex to the ER (Rajamäki & Valkonen, 2009). Restrepo-Hartwig & Carrington (1994) could demonstrate that 6K2 is able to prevent the NIa-Pro nuclear localization when it is bound to NIa-Pro, which is important as an regulatory function in genome replication. Furthermore, an involvement of the protein in symptom induction (Spetz & Valkonen, 2004) and long-distance movement (Rajamäki & Valkonen, 1999) has been demonstrated.

The **VPg** has essential functions in all critical steps of the viral infection cycle. Additionally to its involvement in genome replication, it is required for movement and virulence (Grzela *et al.*, 2008) and was demonstrated to interact with different host proteins (Wittmann *et al.*, 1997; Robaglia & Caranta, 2006). A recent study revealed a possible function of VPg as an auxilliary factor involved in interference of RNA gene-silencing by potyviruses (Rajamäki & Valkonen, 2009).

NIa-Pro represents the key proteinase in potyviral polyprotein processing (Merits *et al.*, 2002; Adams *et al.*, 2005a). Beside this, the protein has non-specific desoxyribonulease (DNase) activity (Anindya & Savithri, 2004). Although NIa-Pro is required for genome replication in the cytoplasm of infected cells, the majority of NIa-Pro molecules are localized to the nucleus of infected cells by two independent nuclear localization signals (NLS) and form typical potyviral nuclear inclusions (Hong & Hunt, 1996; Rajamäki & Valkonen, 2009).

Since it functions as RdRp, the **NIb** is the main protein in virus replication (Hong & Hunt, 1996). As well as NIa-Pro, NIb contains NLS and forms nuclear inclusions (Li *et al.*, 1997; Rajamäki & Valkonen, 2009).

CP as structural protein primarily participates in encapsulation of the viral genome. Additionally, it is involved in vector transmission (Atreya *et al.*, 1991), cell-to-cell as well as long-distance movement. Furthermore, due to an identified interaction between NIb and CP, CP is suggested to play a role in regulation of viral RNA synthesis (Mahajan *et al.*, 1996; Urcuqui-Inchima *et al.*, 2001).

Since **P3N-PIPO** was very recently identified (Chung *et al.*, 2008) not much research about this protein has been conducted yet. At least a participation in cell-to-cell-movement (Wei *et al.*, 2010b) was demonstrated.

A limited number of proteins have to provide all the required functions for establishing a viral infection *in planta*. Firstly, this is ensured by multifunctionality of some proteins. And secondly, as already mentioned above for single proteins, interactions between the proteins play a pivotal role for fulfilling all steps of a virus infection cycle. The next chapters will deal with investigations of protein-protein interactions from a general point of view up to examples for potyviruses.

1.2 Investigation of protein-protein interactions

Proteins are involved in almost all processes in living cells by interacting with other molecules, which include nucleic acids, lipids, carbohydrates or especially other proteins. Protein-protein interactions play a key role in many biological processes. Consequently, the identification and characterization of these interactions and their networks (‘interactome’) is crucial to the understanding of their role in cellular

processes on a molecular and biophysical level (Piehler, 2005; Shoemaker & Panchenko, 2007). Moreover, the identification of protein interactions is often an initial step to reveal the function(s) of a certain protein. Therefore, protein interaction maps provide an overview of the complex and diverse relationships among proteins and enable first hypotheses about their functions, assembly, regulation or modifications (Uetz *et al.*, 2004). Referring to plant viruses, the understanding of the molecular biology of viruses, which includes the functions of the proteins encoded by the viral genomes and the interplay between proteins, is important and necessary for the control of viral spread and development of antiviral strategies (Urcuqui-Inchima *et al.,* 2001).

The study of the molecular biology of single organisms lead to projects of high impact dealing with the development of complete maps of cells by collecting functional genomics data. In addition to information on the genome sequence and the genome expression, these data include information on localization, structure, function, post-translational modifications or especially interactions of proteins. The assembled data should enable a molecular cartography of organisms, e.g., as it was started for the budding yeast *Saccharomyces cerevisiae* several years ago (Bader *et al.*, 2003b). Furthermore, the abundance of complete or partial interaction maps for different organisms as well as human pathogenic viruses lead to the development of protein-protein interaction databases, among them the BIND ('Bimolecular Interaction Network Database'; Bader *et al.*, 2003a) and the DIP ('Database of Interacting Proteins'; Salwinski *et al.*, 2004). These databases deliver information about single proteins and their interaction partners. However, until now the databases contain only little data about viruses, especially plant viruses. The first step to extend knowledge on possible protein-protein interactions comprises the establishment of an appropriate method and therefore, the next chapters will introduce to this topic.

1.2.1 Methods for the detection of protein-protein interactions

A multitude of different technologies have been developed over the last decades for the identification and analysis of protein-protein interactions, which include *in vitro* as well as *in vivo* methods. Thereby, each method has certain advantages, but also different limitations. Furthermore, methods may vary in their sensitivity, specificity, and ability to detect interactions of differing affinity. As a consequence, the choice of a suitable

method is crucial for a specific investigation. Moreover, it is important to decide whether the focus of investigations is laid on the interaction of single protein pairs or the identification of protein complexes before choosing a method (Shoemaker & Panchenko, 2007; Lalonde *et al.*, 2008). The following sections provide a short insight into current methods for the detection and investigation of protein-protein interactions, and finally deliver detailed description of the two methods, which are in the focus of this work. Extensive overviews of these and further methods, as well as detailed comparisons or information about possibilities and limitations of the certain methods were summarized in several review articles (Phizicky & Fields, 1995; Hink *et al.,* 2002; Causier, 2004; Piehler, 2005; Bhat *et al.*, 2006; Shoemaker & Panchenko, 2007; Lalonde *et al.*, 2008; Morell *et al.*, 2009).

The classical biochemical techniques for detecting protein-protein interactions *in vitro* are co-immunoprecipitation (Co-IP; Masters, 2004) and glutathion-S-transferase (GST) pull-down assay (Phizicky & Fields, 1995). Both are based on an affinity purification of a bait protein. Proteins, which are bound to the bait protein are eluted and subsequently analyzed, e.g., for an identification by mass spectrometry (Piehler, 2005). A similar procedure is the tandem affinity purification (TAP; Rigaut *et al.*, 1999; Puig *et al.*, 2001), which uses a TAP-tag bound to the bait protein for affinity purification. Furthermore, blot or gel overlay assays are widely used for interaction studies (Hall, 2004). This method is based on a fractionation of proteins by SDS-PAGE, which is followed by blotting of the proteins to a membrane and incubating with a probe of interest. The probe, which is typically a protein, may be radiolabeled or visualized with a specific antibody.

Since these methods require lysis of the cells they can deliver only incomplete information about potential protein-protein interactions in living cells (Citovsky *et al.*, 2006). This is generally a limitation of *in vitro* methods, as they cannot reflect the natural conditions. Therefore, a number of *in vivo* technologies achieved high popularity as the yeast two-hybrid system (Fields & Song, 1989) and the bimolecular fluorescence complementation (Hu et al., 2002), which are introduced in the next chapters.

1.2.1.1 Yeast two-hybrid (YTH) systems

The most popular and often applied method for the investigation of protein-protein interactions *in vivo* is the YTH assay (Fields & Song, 1989). It enables the identification of binary interactions between known proteins or new interacting proteins by screening expression libraries, as well as the determination of protein domains involved in a particular interaction (Toby & Golemis, 2001; Causier, 2004). This is a significant advantage over other methods and the main reason why the system enjoy great popularity. During the last years the YTH technology has been continuously developed and meanwhile different systems are commercially available.

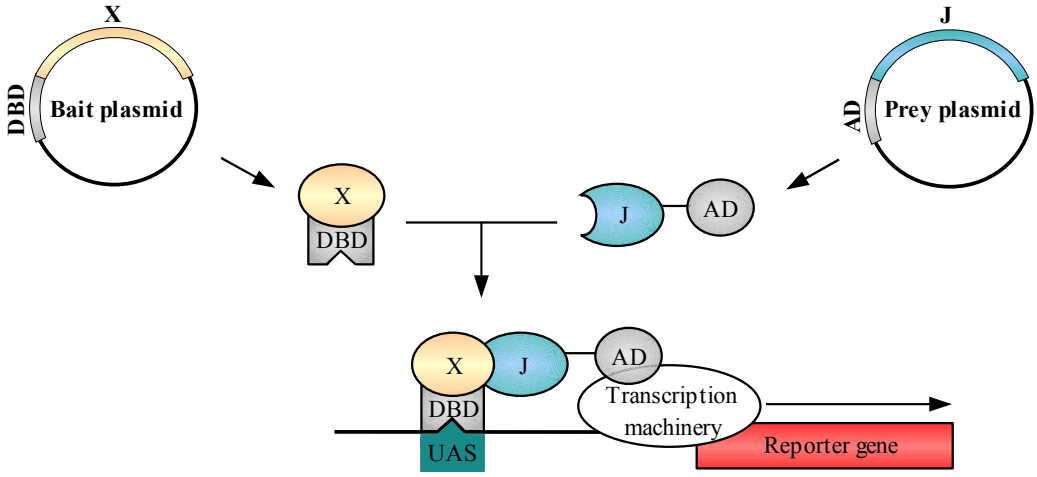

Fig. 4: The principle of the YTH assay. Two plasmids are constructed, which encode either a fusion of the protein X and the DNA-binding domain (DBD) of a transcriptional activator or a fusion of the protein J and the activation domain (AD) of the transcriptional activator. Subsequently, the plasmids are introduced into an appropriate yeast strain. When proteins X and J physically interact, DBD and AD are brought into tight contact to reconstitute a functional transcription factor. This binds to upstream activation sequences (UAS) in the promoter of the reporter gene(s) and thus activates their expression (modified after Causier, 2004).

The basic idea of the system derives from the fact that many eukaryotic transcription activators, which represent proteins involved in gene regulation, are of a modular structure and can be separated into two functional domains, namely a DNA-binding domain (DBD) and an activating domain (AD) (Bram *et al.*, 1986; Keegan *et al.*, 1986; Chien *et al.*, 1991). The DBD acts by binding to upstream activating sequences (UAS) of the promoter region of certain genes within the genome of an organism, whereas the AD recruits the transcription machinery to activate gene transcription. Since none of both domains is able to activate transcription on its own, spatial proximity of the two domains is required for this. In a YTH assay these two domains AD and DBD of a certain transcription activator are expressed in special yeast strains from plasmids as

translational fusions with two proteins of interest, for which an interaction should be verified. The protein X, fused to the DBD is called the 'bait', whereas the protein J, which is fused to the AD, is called the 'prey'. Upon interaction between proteins X and J, the AD and the DBD are brought into tight contact and a functional transcription activator is reconstituted inducing the transcription of the respective reporter gene (Fields & Song, 1989; Causier, 2004; Fig. 4).

The original system developed by Fields & Song (1989) utilizes the yeast GAL4 transcription factor. An alternative system is based on the DBD of the bacterial repressor protein LexA in combination with the *Escherichia coli* B42 AD, giving the LexA-system, also called the 'yeast interaction trap' (Ruden *et al.*, 1991; Gyuris *et al.*, 1993). Further modifications of and improvement over the original system concern the amount of reporter genes used for the identification of protein-protein interactions. As the original system relied on one single reporter gene, current systems utilize several reporter genes, auxotrophic reporters (Feilotter *et al.*, 1994; James *et al.*, 1996; Aho *et al.*, 1997; Causier & Davies, 2002) as well as reporter genes, which enable colorimetric assays or fluorescence detection (Aho *et al.*, 1997; Cormack *et al.*, 1998; Causier, 2004), at once. This provides a more stringent assay and should help to increase specificity (Causier, 2004).

A multitude of studies were performed using the YTH technology. However, although the system allow fast investigation of protein interactions it has certain drawbacks, which are continuously discussed by the users. It indeed enables the investigation of protein-protein interactions in a cellular context of eukaryotic cells. But since not only yeast proteins are investigated with this technology, yeast cells do not represent natural conditions, e.g., for plant or mammalian proteins. The main problem of the system is the consistently occurrence of so called ´false negative` and `false positive' interactions. These may have different causes and some should be explained here. The system depends on proteins, which localize to the yeast cell nucleus to activate transcription (Golemis *et al.*, 1999; Causier & Davies, 2002). Proteins with strong signals for localization to other parts of the cell, e.g., nuclear export signal (NES) or highly hydrophobic domains (integral membrane proteins) cannot activate transcription in the nucleus (van Criekinge & Beyaert, 1999; Causier, 2004). This may result in false negative interactions. 'False positives' include proteins that randomly interact in a YTH assay, but are normally expressed in different parts of the cell (Colas & Brent, 1998) or

proteins that activate transcription on their own without a physical interaction with the second protein. Moreover, proteins that are not stable expressed, toxic to yeast cells or repressors of transcription may be critical for investigation in YTH approaches (Causier & Davies, 2002).

The YTH technology was steadily improved resulting in a number of different yeast-based methods to circumvent a number of limitations. Among these is the split-ubiquitin system for the study of interactions between membrane proteins (Johnsson & Varshavsky, 1994; Stagljar *et al.*, 1998) or the Sos-recruitment system, which enables the investigation of protein interactions in the cytoplasm (Aronheim *et al.*, 1997). Furthermore, the systems were adapted to bacterial or even mammalian cells giving the bacterial two-hybrid (Joung *et al.*, 2000) and mammalian two-hybrid systems (Lee & Lee, 2004). However, these bacterial or mammalian systems do not reach the popularity of the standard system (Piehler, 2005) and often are not suitable for plant research (Causier & Davies, 2002).

Since its development by Fields and Song (1989), the YTH has been the method of choice for protein-protein interaction studies. Meanwhile, other methods have been established. Consequently, due to the described characteristics of YTH systems, interactions identified by YTH require the confirmation by other techniques, which reflect a more natural environment of the proteins of interest (Immink *et al.*, 2002; Uetz *et al.*, 2004). A system, which can afford this, is the bimolecular fluorescence complementation.

1.2.1.2 Bimolecular fluorescence complementation (BiFC)

BiFC analysis has become a powerful alternative method for studying protein-protein interactions in living cells and belongs to the fluorophore-based methods. For a better understanding of the method, a short insight into fluorescent proteins dealing with their main attributes is provided in the next section.

Fluorescent proteins are widely used as reporter genes in genomic research. They are introduced into and expressed in cells of the test organism alone or as fusions with proteins of interest and enable a visualization of biological processes on a subcellular level *in vivo,* e.g., reflect the level of gene expression or the subcellular localization of fused proteins of interest (Tsien, 1998). This does not require exogenous substrates or

co-factors as in case of, e.g., *E. coli* ß-Glucuronidase (GUS; Cody *et al.*, 1993; Baulcombe *et al.*, 1995; Chudakov *et al.*, 2005). Upon excitation with light of a certain wavelength fluorescence can be detected, typically on cellular level by fluorescence microscopy. The most famous fluorescent protein is the green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* (Shimomura *et al.*, 1962). Since its discovery several useful mutants have been generated (Chudakov *et al.*, 2005), as the blue fluorescent protein (BFP), a cyan fluorescent protein (CFP; Heim *et al.*, 1994) or the yellow fluorescent protein (YFP; Ormö *et al.*, 1996), which differ in their spectral as well as biochemical properties. Furthermore, the red fluorescent protein from the coral *Discosoma striata* (DsRed; Matz *et al.*, 1999; Baird *et al.*, 2000) and its variants (Shaner *et al.*, 2004) covers the red part of the visible spectrum. The fluorescent proteins are composed of approximately 240 aa with a characteristic folding. Proteins are comprised of β-barrels forming some kind of cylinder with an α-helix in the middle. This α-helix bears the chromophore, which is build up by special folding of three fluorophore-dependent aa (Yang *et al.*, 1996) and is responsible for emitting fluorescence.

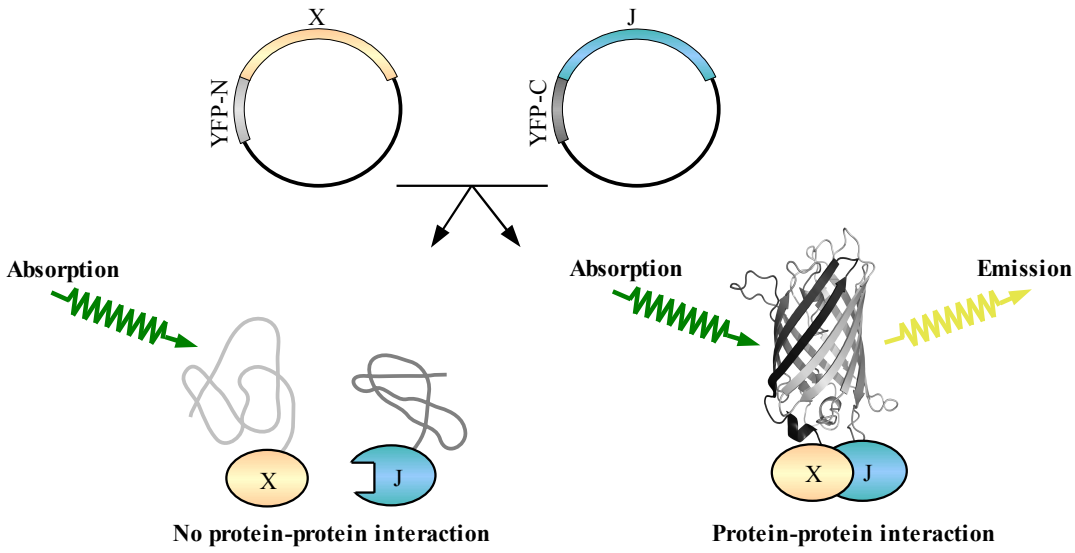

Fig. 5: The principle of the BiFC assay for the detection of protein-protein interactions in living cells, exemplified by a YFP. Proteins of interest, X and J, are expressed as fusions with the non-fluorescent fragments of a YFP from plasmids in the appropriate test organism, e.g., *in planta*. In the absence of an interaction between the both proteins, the YFP fragments remain non-functional, whereas upon interaction of X and J a functional fluorophore is reconstituted. In the latter case emission of fluorescence is produced upon excitation with an appropriate wavelength (modified after Bhat *et al.*, 2006).

For BiFC such a fluorescent protein is divided on nucleotide level into two parts, encoding an N-terminal fragment and a C-terminal fragment, which cannot emit light of

a characteristic wavelength on their own. Only when both fragments are brought into tight contact, a functional fluorophore is reconstituted and fluorescence is detected. Upon physical interaction between two proteins of interest, expressed as translational fusions with the non-fluorescent fragments, the association of the fluorescent protein is initiated and light of a characteristic wavelength emitted (Hu *et al.*, 2002; Fig. 5).

Over the past years the BiFC assay has been used to detect and visualize protein interaction in numerous species from many different phyla (Kerppola, 2008). The very first BiFC system, based on an enhanced YFP was developed to visualize subcellular localization of activator protein (AP-1) dimers in mammalian cells. However, the system not only revealed subcellular localization, but also enabled visualization of interaction between these activator proteins and certain transcription factors (Hu *et al.*, 2002; Hu & Kerppola, 2003). Meanwhile, the system has been applied to numerous other organism, including bacteria (Atmakuri *et al.*, 2003; Magliery *et al.*, 2005), yeast (Blondel *et al.*, 2005; Cole *et al.*, 2007; Sung & Huh, 2007), filamentous fungi (Hoff & Kück, 2005), the fruit fly *Drosophila melanogaster* (Benton *et al.*, 2006), the nematode *Caenorhabditis elegans* (Hiatt *et al.*, 2008) or even embryos of the claw-toed frog *Xenopus laevis* (Saka *et al.*, 2007) to mention only some early reports. In plant cells, BiFC was first performed in transfected *Arabidopsis thaliana* protoplasts (Walter *et al.*, 2004). At the same time initial approaches with *Agrobacterium tumefaciens*-mediated protein expression in epidermal leaf cells of *Nicotiana benthamiana* and *A. thaliana* were performed (Bracha-Drori *et al.*, 2004), allowing new proteomic approaches in plant research.

Consequently, BiFC technology can be described as generally applicable for visualizing protein-protein interactions in theoretically all cell types or organisms that are able to express proteins that are fused to the fragments of the fluorescent proteins. Significant advantages of this system are the high specificity and high stability of the reconstituted chromophore complex and its intrinsic fluorescence, thus avoiding the use of exogenous reagents or dyes for detection (Hu & Kerppola, 2002; Kim *et al.*, 2007). Therefore, any standard fluorescence microscope can be used and the assay can be performed without any additional and expensive instrumentation. Furthermore, it enables a fast and direct real-time visualization of the protein complex in the normal cellular environment, which is one of the most important advantages over *in vitro* methods or the YTH system. However, although BiFC enables the detection of the protein complexes, it does not

provide the potential for real-time detection of complex dynamics, that means real-time complex formation and dissociation. A more detailed overview on the BiFC system including the information about experimental strategies, controls and interpretation of BiFC results, as well as possibilities and limitations of the technology are presented in articles by Citovsky *et al.* (2008) and Kerppola (2008).

The spectral possibilities to perform BiFC assays is enormous. As described above, the first BiFC based on an enhanced YFP (Hu & Kerppola, 2002). Beside YFP several other fluorescent proteins, as GFP, BFP, CFP and further variants thereof have been reported to be useful for this technology (Weinthal & Tzfira, 2009). Consequently, also red fluorescent proteins (RFP) were utilized to establish BiFC systems adding red to the spectrum for BiFC. The first RFP-based system was developed by Jach *et al.* (2006) to investigate dimerization of plant transcription factors in leaf epidermal cells. As DsRed and most of its variants are known to tetramerize (Bevis & Glick, 2002) Jach *et al.* (2006) generated different improved variants of the monomeric red fluorescent protein 1 (mRFP1; Campbell *et al.*, 2002), from which the mRFP1 with an Q to T aa substitution at position 66 (Q66T) allowed to establish a red BiFC. Meanwhile, this mRFP1-Q66T BiFC system was followed by further RFP-based systems. The mCherry system (Fan *et al.*, 2008) added an RFP to BiFC, with a shorter maturation time than the mRFP1-Q66T BiFC. Furthermore, a recently published far-red system (Chu *et al.*, 2009) using mLumin as reporter, enabled a red BiFC under higher temperatures up to 37°C in comparison to the other ones only working on lower temperatures (< 30 °C).

New variants of the BiFC technology are based on a complementation between fragments of different fluorescent proteins, which enable a simultaneous visualization of multiple protein interactions in the same cell (multicolor fluorescence complementation analysis; Hu & Kerppola, 2003; Kodama & Wada, 2009). Moreover, with a combination of BiFC and a further fluorophore-based method for the detection and visualization of protein-protein interactions, the Förster or fluorescence resonance energy transfer (FRET; Gordon *et al.*, 1998), tri- or oligomeric protein complexes can be investigated and visualized (Shyu & Hu, 2008). FRET is based on an energy transfer between a donor fluorescent molecule, which is excited with an appropriate monochromatic light, and an acceptor chromophore when these two are brought into close proximity (Cardullo, 2007). This is the case upon physical interaction of two proteins fused to the two different fluorescent proteins (Gordon *et al.*, 1998; Gadella *et al.*, 1999; Periasamy,

2001). A variety of FRET is the bioluminescence resonance energy transfer (BRET; Ciruela, 2008), only distinguishing from the former one by the initial activator of the system. BiFC in combination with FRET or BRET provides an opportunity to analyze protein-protein interactions in detail and to detect complete complexes. However, FRET and BRET technologies require sophisticated and expensive equipment as well as complex data analysis and intensive methodical training (Hink *et al.*, 2002; Walter *et al.*, 2004), and thus are not applicable for a fast and straightforward detection of protein-protein interactions.

1.2.2 Potyviral protein-protein interactions

Several interactions between potyviral proteins have been analyzed during the last decades predominantly using different YTH systems (Hong *et al.*, 1995; Li *et al.*, 1997; Guo *et al.*, 1999; Merits *et al.*, 1999; Urcuqui-Inchima *et al.*, 1999; Choi *et al.*, 2000; López *et al.*, 2001; Roudet-Tavert *et al.*, 2002; Yambao *et al.*, 2003; Seo *et al.*, 2010). Even complete interaction maps for the ten potyviral proteins, excluding P3N-PIPO, of a number of potyviruses were generated by systematic two-hybrid testing of protein pairs for interactions, typically in a kind of array format (Uetz *et al.*, 2004). These investigations were carried out for the potyviruses PVA, *Pea seed-borne mosaic virus* (PSbMV) (Guo *et al.*, 2001), *Papaya ringspot virus* strain P (PRSV-P) (Shen *et al.*, 2010), *Shallot yellow stripe virus* strain O (SYSV-O) (Lin *et al.*, 2009) and different strains of SMV, SMV-G7H and SMV-P (Kang *et al.*, 2004; Lin *et al.*, 2009). However, so far, no interaction map for PPV proteins has been developed. López *et al.* (2001) examined the CI of PPV with respect to self-interaction and interaction with five other PPV proteins, but failed to detect interactions of the full-length CI. Results of these investigations gave a first, but incomplete insight into the interaction network of PPV proteins.

Focusing on complete protein interaction maps and further protein interaction studies within the genus *Potyvirus*, YTH experiments with different viruses have not given necessarily consistent results for interactions between homologous viral proteins. Some examples are given here: An interaction between CI and NIa was reported for SMV-P, SYSV-O (Lin *et al.*, 2009) and PRSV-P (Shen *et al.*, 2010), but not for SMV-G7H (Kang *et al.*, 2004), TEV (Li *et al.*, 1997) or PPV (López *et al.*, 2001). And also the

interaction between HC-Pro and CP, which was often analyzed and described as essential for aphid-transmission (Atreya & Pirone, 1993; Blanc *et al.*, 1997, 1998; Peng *et al.*, 1998), was confirmed in YTH assays, e.g., for SMV-G7H (Kang *et al.*, 2004; Seo *et al.*, 2010) and *Lettuce mosaic virus* (LMV) (Roudet-Tavert *et al.*, 2002), but was neither detected for PVA and PSbMV (Guo *et al.*, 2001) nor for SMV-P (Lin *et al.*, 2009) and PRSV-P (Shen *et al.*, 2010). Therefore, the diversity of interactions seems to be very high and it is difficult to verify if this results from YTH methodological limitations or if viruses of the genus *Potyvirus* really show such inconsistent protein interaction behaviour.

Additionally, there are some very recent reports about investigation of potyviral protein interactions using BiFC subsequent to YTH assays. Especially HC-Pros of different potyviruses were in the focus of these studies. The HC-Pro of TuMV was investigated with regard to a mapping of self-interacting domains and subcellular localization of the protein (Zheng *et al.*, 2010). Seo *et al.* (2010) carried out interaction studies with the CP and HC-Pro of SMV including mutational analysis of the two proteins. In both studies for single tested protein combinations no interaction was detected in a YTH assay whereas a BiFC assay with the same protein combinations delivered positive results. These inconsistent results raise the question of whether a yeast system or a system performed under natural conditions reflects the real occurring protein interactions. However, complete interaction maps for potyviruses have not yet been generated using the BiFC method. Until now, only one single interaction map for the proteins of a plant virus was reported, namely for *Potato yellow dwarf virus* (PYDV), the type member of the genus *Nucleorhabdovirus* (Bandyopadhyay *et al.*, 2010).

1.3 Objectives

PPV is a virus of great economic impact, causing significant losses in *Prunus* cultures world-wide. The virus can decrease the yield up to a total loss. The cost for sharka management including costs for eradication programmes or sanitary controls world-wide during the last three decades have been estimated up to 10,000 million Euros (Cambra *et al.*, 2006). To gain continuously more knowledge about the virus is an important step in future development of antiviral strategies aiding in disease control.

Protein-protein interactions play important roles during the virus infection cycle. Therefore, the identification of viral protein interactions is crucial to the understanding of the molecular biology of viruses. Preliminary studies of PPV protein interactions focused on single proteins giving only little information about the protein interaction network of this virus. A complete interaction matrix for the proteins of this virus has not yet been developed, whereas for a number of other potyviruses such interaction maps have been generated using different YTH systems. However, results of these studies are not consistent among the proteins of potyviruses.

Motivated by the reported facts the objectives of this work were (a) to optimize an existing BiFC system to enable the investigation of protein interactions *in planta* and (b) to advance knowledge on the interaction network of PPV proteins with this optimized system in *N. benthamiana.*

For the optimization, a recently developed BiFC system, which utilizes an mRFP as reporter was chosen. For the implementation of the system in plant viral interaction studies, BiFC plasmids were modified in different steps. Subsequently, appropriate interacting and non-interacting controls, consisting of PPV_CP and mutants thereof, were developed. Finally, different proteins of viruses from other genera were used to validate the system.

The second part of the work focused on the implementation of the optimized BiFC-system for the detection of PPV proteins *in planta*. Employing this BiFC system, a complete interaction map for ten plum pox potyviral proteins, excluding P3N-PIPO, was generated. This constitute the first BiFC-based interaction map for a member of the genus *Potyvirus*. Additionally, due to the capability of the system to visualize protein interactions directly *in planta*, the system was demonstrated to enable a localization of these interactions. Referring to this, experiments were carried out with PPV_P3N-PIPO with regard to its involvement in potyviral cell-to-cell movement. Last but not least the system was used to investigate single interactions of PPV_HC-Pro and TuMV_HC-Pro as well as mutants thereof to validate differences in the interaction behavior of homologous proteins of different potyviruses and to exclude methodical insufficiency.

All together the study should on the one hand provide a suitable and fast system for the detection and visualization of protein interactions between known proteins *in planta.* On the other hand this work should help to understand the interactions occurring between PPV proteins *in planta* to give hints for the interplay during the virus infection cycle.

2 An optimized mRFP-based bimolecular fluorescence complementation system for the detection of protein-protein interactions *in planta*

2.1 Abstract

An existing bimolecular fluorescence complementation (BiFC) system, based on a monomeric red fluorescent protein (mRFP), has been optimized for the investigation of protein-protein interactions *in planta*. The expression plasmids, encoding the N-terminal amino acids (aa) 1-168 and the C-terminal aa 169-225 of the mRFP, allow N- or C-terminal fusion of a split mRFP, with the genes of interest. Two major improvements over the original vectors have been made. Firstly, the coding sequence of a GGGSGGG-linker has been integrated between mRFP sequences and the genes of interest. Secondly, a modified mini binary vector (~ 3.5 kb) was introduced as the backbone for the plant expression plasmids. Based on the results of yeast two-hybrid studies with plant viral proteins, interaction of viral proteins was tested in *Nicotiana benthamiana* plants and monitored by confocal laser scanning microscopy (CLSM). *Plum pox virus* coat protein and mutants thereof served as controls. The system was validated using the N-protein of *Capsicum chlorosis virus* for which a self-interaction was shown for the first time, the *Tobacco mosaic virus* coat protein and BC1 and BV1 of the *Tomato yellow leaf curl Thailand virus*. This optimized BiFC system provides a convenient alternative to other BiFC, as well as yeast two-hybrid assays, for detecting protein-protein interactions.

Keywords: Bimolecular fluorescence complementation (BiFC), protein-protein interaction, red fluorescent protein, *Plum pox virus*, *Capsicum chlorosis virus*

2.2 Introduction

Protein-protein interactions play significant roles in many cellular processes. Even for processes of the plant viral infection cycle, e.g., the formation of the replication complex, cell-to-cell movement and the assembly of virions, they are essential. Furthermore, the study of protein interactions is essential for the understanding of protein function. Many examples of plant viral protein-protein interactions have been reported, and complete interaction maps for the proteins of certain plant viruses, e.g., some potyviruses, have been developed (Guo *et al.*, 2001; Kang *et al.*, 2004; Lin *et al.*, 2009; Shen *et al.*, 2010) demonstrating a high diversity of interactions.

In general, the determination of protein interactions has been performed using *in vitro* approaches, such as gel overlay assays (Hall, 2004) and co-immunoprecipitation ('pulldown assay') (Masters, 2004; Phizicky & Fields, 1995), or by *in vivo* techniques, namely the yeast two-hybrid system (Fields & Song, 1989). The latter method is the most popular and is based on transcriptional activation of a reporter gene in the yeast nucleus. However, this method has certain limitations, which includes the use of yeast cells as heterologous test organisms; the requirement for the nuclear import of proteins; and the generation of false positive results by proteins that activate transcription of the reporter gene in the absence of any interaction partner.

All of these techniques offer a chance to detect physical protein interactions, but they neither represent the dynamics of the interaction nor reflect correct modifications or subcellular localization of proteins *in vivo* in real time (Stolpe *et al.*, 2005). The system of choice to detect and visualize protein-protein interactions in real time in living cells is fluorescence resonance energy transfer (FRET) (Gordon *et al.*, 1998), but this requires specialized and expensive equipment for fluorescence lifetime imaging, as well as special algorithms for data analysis (Bhat *et al.*, 2006; Sekar & Periasamy, 2003), and thus represents a very elaborate and time-consuming method.

Bimolecular fluorescence complementation (BiFC) analysis has become a powerful alternative method for studying protein-protein interactions in living cells. BiFC is based on the expression of two proteins of interest fused to the non-fluorescent N-terminal or C-terminal fragment of a fluorescent protein, e.g., yellow fluorescent protein (YFP). Only when both fragments are brought into tight contact by physical interaction of the two fused proteins is a functional fluorescent protein reconstituted and

fluorescence detected. This system was first developed to detect protein-protein interactions in mammalian tissue culture cells (Hu *et al.*, 2002; Hu & Kerppola, 2003), but it has also been applied to other organisms, including bacteria (Atmakuri *et al.*, 2003), yeast (Blondel *et al.*, 2005; Sung & Huh, 2007) and a filamentous fungus (Hoff & Kück, 2005). In plant cells, BiFC was first performed in transfected *Arabidopsis thaliana* protoplasts (Walter *et al.*, 2004) and with *Agrobacterium tumefaciens*-mediated protein expression in epidermal leaf cells of *Nicotiana benthamiana* and *A. thaliana* (Bracha-Drori *et al.*, 2004). Significant advantages of this system are the high specificity and high stability of the reconstituted chromophore complex and its intrinsic fluorescence, thus avoiding the use of exogenous reagents for detection (Hu *et al.*, 2002; Kim *et al.*, 2007). Furthermore, it enables a fast and direct real-time visualization of the protein complex under physiological conditions, which is one of the most important features in comparison to *in vitro* methods or the yeast two-hybrid system.

A previous study described the use of BiFC for the detection of protein-protein interactions in plant cells using a modified monomeric red fluorescent protein (mRFP), mRFP1-Q66T (Jach *et al.*, 2006), in contrast to the original system described by Hu *et al.* (2002) using a YFP. The present study describes further optimization of the mRFP1-Q66T system for the investigation of plant viral protein interactions *in planta*. To enable the detection of protein-protein interactions using an mRFP-based BiFC system, two different sets of expression plasmids were generated, allowing interaction studies in transiently transformed *N. benthamiana* cells to be performed. Each plasmid set enables the expression of the proteins of interest fused to the N-terminal 168 amino acids (aa) or to the C-terminal aa 169-225, respectively, of mRFP1-Q66T (kindly provided in plasmids pBat_TL-smRFP_N and pBat_TL_smRFP_C by J. Uhrig, University of Cologne, Germany). Moreover, the plasmids contain sequences encoding either a c-myc or hemagglutinin (HA) tag, which allow the detection of fusion proteins in immunoblot analyses. One important modification of the system is the addition of a linker sequence encoding 7 aa (GGGSGGG) (Schwartz *et al.*, 2004) inserted between the genes of interest (GOI) and the mRFP fragments. A similar linker was described in the literature (Bracha-Drori *et al.*, 2004; Fan *et al.*, 2008) for BiFC and allows maximal flexibility of the fusion proteins during complex formation (Kerppola, 2006). A further feature of the modified system is that the two plasmid sets enable the fusion of the proteins of interest to either to the N- or C-terminal domain of the mRFP fragments.

The modified system was used to investigate and visualize homodimerization of viral proteins from viruses deriving from different genera: the coat protein (CP) of *Plum pox virus* (PPV, Potyvirus), the N-protein of *Capsicum chlorosis virus* (CaCV, Tospovirus) and the CP of *Tobacco mosaic virus* (TMV, Tobamovirus). Self-interaction of potyviral CPs has already been demonstrated for several potyviruses in yeast two-hybrid assays (Guo *et al.*, 2001; Kang *et al.*, 2004, 2006; Lin *et al.*, 2009; Shen *et al.*, 2010). And self-interaction of the N-protein of *Tomato spotted wilt virus*, a Tospovirus, has been demonstrated also using a yeast two-hybrid system (Uhrig *et al.*, 1999) and by FRET in baby hamster kidney (BHK21) cells (Snippe *et al.*, 2005). Furthermore, particle assembly of TMV particles requires the interaction of the CP subunits (Bendahmane *et al.*, 1999; Namba *et al.*, 1989), and therefore, TMV_CP is also expected to interact with itself. All of the above mentioned proteins should enable a suitable demonstration of the functionality of the system.

Additionally, different PPV_CP-mutants, similar to the CP mutants of the *Soybean mosaic virus* strain G7H (SMV-G7H; Kang *et al.*, 2006), were generated and tested for their ability to self-interact and used to establish an appropriate interacting and non-interacting control for the system. The proteins BC1 and BV1 of *Tomato yellow leaf curl Thailand virus* (TYLCTHV, Begomovirus) were also analyzed to validate the BiFC system further. Frischmuth *et al.* (2004) performed interaction studies with BC1 and BV1 of *Abutilon mosaic virus* (AbMV), another Begomovirus, using the yeast two-hybrid system, revealing self-interaction of BC1 but no self-interaction of BV1 or interaction between BC1 and BV1.

2.3 Materials and methods

2.3.1 Construction of BiFC plasmids

To generate modified BiFC plasmids, pBat_TL-smRFP_N and pBat_TL_smRFP_C (Jach *et al.*, 2006), which contain the coding sequences for the N-terminus (mRFPN: aa 1-168) and the C-terminus (mRFPC: aa 169-225) of the improved mRFP (mRFP1-Q66T), were used. The enhanced Cauliflower mosaic virus 35S promoter (pe35S), fused to the translation enhancer (TL) of the Tobacco etch virus was released from pBat_TL-smRFP_N with *Eco*RV/*Bgl*II and subcloned into modified pRT100 (Toepfer *et al.*, 1987), resulting in plasmid pe35S-TL. The sequences encoding myc-mRFPN and HA-

mRFPC, including downstream 35S polyadenylation signals, were retrieved from the original plasmids by digestion with *Spe*I/*Hin*dIII and integrated into pe35S-TL resulting in pe35S-TL-myc-mRFPN-35SpA and pe35S-TL-HA-mRFPC-35SpA, respectively.

Tab. 1: Oligonucleotides

	Primer	Sequence (5` → 3`) [a b]	Restriction sites
PCR fragment			
RBS-CP-Linker / -attR2	RBS	ATAGATCT**TCGAAGGAGATATAACAATG** GGATCC*GCT*GACGAAAGAGAAGACGAG	*Bgl*II *Bam*HI
	Linker	TCACTAGT**ACCTCCACCAGATCCACCTCC** GTCGAC*CAC*TCCCCTCATACCGAGGAGGTTG	*Spe*I *Sal*I
	attR2	TCACTAGT**GACCACTTTGTACAAGAAAGCT** **GA**GTCGACCACTCCCCTCATACCGAGGAGGT	*Spe*I *Sal*I
PPV_CP	CPY2Hs	AAAGGATCC*GCT*GACGAAAGAGAAGACGAG	*Bam*HI
	CPY2Has	TTTCTCGAG*CAC*TCCCCTCATACCGAGG	*Xho*I
GUS	BiFC-GUS_s	AAGGATCC*ATG*TTACGTCCTGTAG	*Bam*HI
	BiFC-GUS_as	AACTCGAG*TTG*TTTGCCTCCCTG	*Xho*I
CaCV_N	CaCV_N1	AGGATCC*ATG*TCTACCGTCAGGCAACT	*Bam*HI
	CaCV_N2	AGTCGAC*CAC*TTCAATCGATGTACTA	*Sal*I
TMV_CP	TMV_CP1	AGGATCC*ATG*TCTTATAGTATCACTA	*Bam*HI
	TMV_CP2	AGTCGAC*AGT*TGCAGGACTAGAGGTC	*Sal*I
TYLCTHV_BC1	BC1_1	AGGATCC*ATG*GAGTCAGGAACTAACAA	*Bam*HI
	BC1_2	AGTCGAC*TAT*TTGCTTTACATTTGAG	*Sal*I
TYLCTHV_BV1	BV1_1	AGGATCC*ATG*AGAGTTCCTACTAGACGACC	*Bam*HI
	BV1_2	AGTCGAC*TCC*AATGTAGTTCAAATCG	*Sal*I
Site directed mutagenesis fragment			
pGEM®-T Easy_F1	PPV_N1	CTCGAGAAAAATCACTAGTGAATTCG	*Xho*I
	PPV_N2	TGCCTTCAAACGTGGCACTGTA	
pGEM®-T Easy_F3	PPV_C1	CGAAATTATGAAAAAGCATACATGC	
	PPV_CC2	GGATCCTTTAATCGAATTCCCG	*Bam*HI
pCB:myc-mRFPN	2453 (sense1)	AGTGAGCAAAAGTTGATTTCTGAGGAGG	
pCB:HA-mRFPC	2455 (sense 2)	AGTATGTACCCATACGATGTTCCAGATTAC	
	2454 (antisense1)	AGTAGATCCCATTGTTATATCTCCTTCGAAG	
	2456 (sense1)	TCCAAAGTCGACTAAGGATCGATCCTCTAGA GTC	*Sal*I
pCB:mRFPN-*Bam*HI/*Sal*I	2457 (antisense1)	TCCACCTCCACCAGATCCACCTCCCTTCAGC TTCAGCCTCATCTTGA	
pCB:mRFPC-*Bam*HI/*Sal*I	2458 (antisense2)	TCCACCTCCACCAGATCCACCTCCGGCGCCG GTGGAGTGGCGG	

[a] Restriction sites are underlined. First and last codons of the specific sequences are in italics.
[b] RBS, linker and attR2 encoding sequences are in bold.

The coding sequence of the PPV_CP was first PCR amplified from a PPV full-length clone (Maiss *et al.*, 1992) with primers RBS and Linker (Tab. 1). These primers introduced *Bgl*II and *Bam*HI restriction sites, a ribosomal binding site (RBS), a GGGSGGG-linker coding sequence and *Sal*I and *Spe*I sites. Secondly, PCR amplification of the PPV_CP coding sequence was performed with primers RBS (see above) and attR2 (Tab. 1), thus introducing the coding sequence for the rudimentary attachment-site (attR2: SAFLYKVV) of the Gateway®-Cloning system used in the original system. The PCR fragments RBS-PPV_CP-linker and RBS-PPV_CP-attR2 were cloned into the above mentioned plasmids using the introduced *Bgl*II/*Spe*I sites to generate pe35S-CP-linker-myc-mRFPN and pe35S-CP-linker-HA-mRFPC, as well as pe35S-CP-attR2-myc-mRFPN and pe35S-CP-attR2-HA-mRFPC. The complete expression cassettes were digested with *Sna*BI/*Hin*dIII and ligated into the corresponding sites of pBIN19 (Bevan, 1984) resulting in pBIN19:PPV_CP-linker-mRFPN and -mRFPC and pBIN19:PPV_CP-attR2-mRFPN and -mRFPC, respectively.

For comparison of linker- and attR2-constructs, the self-interaction of two different proteins was analyzed: PPV_CP, a viral protein, and *Escherichia coli* β-glucuronidase (GUS). The latter protein is known to form tetramers (Bracey & Paigen, 1987) and is suitable to use as a positive control. The entire coding sequence of GUS was PCR amplified with primers BiFC-GUS_s and BiFC-GUS_as (Tab. 1), digested with *Bam*HI and *Xho*I and inserted in place of PPV_CP in pBIN19:PPV_CP-mRFPN and -mRFPC, as well as in pBIN19:PPV_CP-attR2-mRFPN and -mRFPC digested with *Bam*HI/*Sal*I. The resulting plasmids were designated as pBIN19:GUS-linker-mRFPN and -mRFPC and pBIN19:GUS-attR2-mRFPN and -mRFPC, respectively.

To test autoactivation of mRFP fragments, the PPV_CP coding region in pBIN19:PPV_CP-linker-mRFPN and -mRFPC was replaced by a 17-aa encoding multiple cloning site (MCS), which was removed from pBluescript II KS(+) plasmid using *Bam*HI/*Sal*I and introduced into the plasmids digested with the same enzymes resulting in pBIN19:MCS-mRFPN and pBIN19:MCS-mRFPC (Fig. 1a).

During further optimization, expression cassettes pe35S-CP-linker-myc-mRFPN and pe35S-CP-linker-HA-mRFPC were digested with *Sna*BI/*Hin*dIII and ligated into the corresponding sites of a modified mini binary plasmid pCB301 (Xiang *et al.*, 1999). This new vector should simplify the cloning procedure due to easier handling and higher stability than pBIN19. The resulting plant expression plasmids were designated

pCB:PPV_CP-mRFPN and pCB:PPV_CP-mRFPC. By using *Bam*HI/*Sal*I, the PPV_CP gene was replaced by other GOI, generating the plasmids pCB:GOI-mRFPN and pCB:GOI-mRFPC (Fig. 1b).

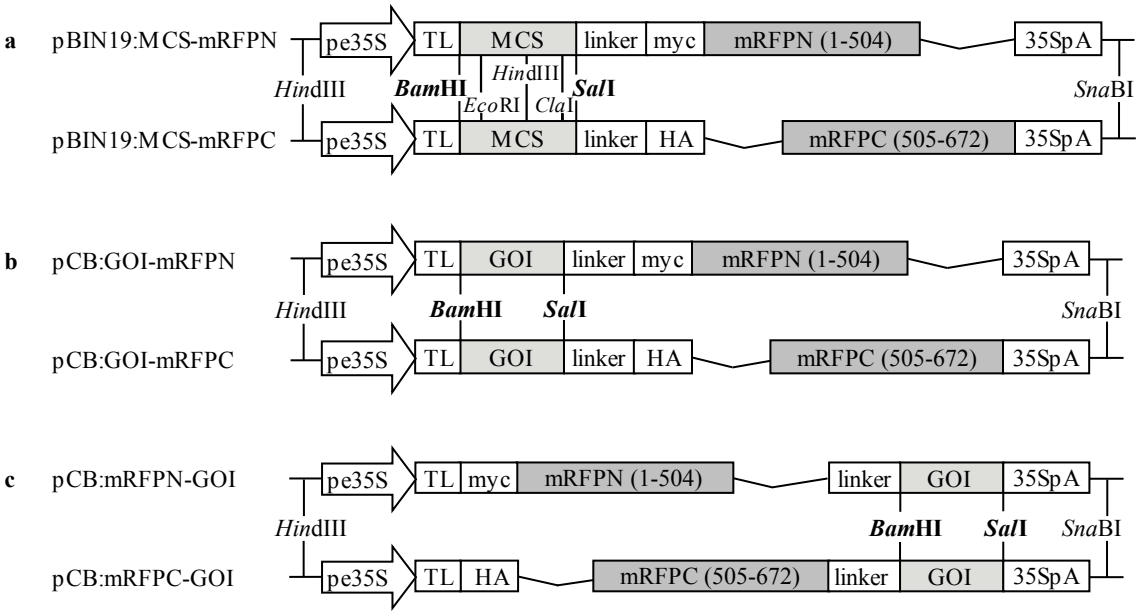

Fig. 1: Schematic view of the expression cassettes of BiFC empty control plasmids (a) and BiFC mini binary plasmids (b and c). A 51-bp MCS was introduced with *Bam*HI/*Sal*I into pBIN19 binary plasmids giving pBIN19:MCS-mRFPN and pBIN19:MCS-mRFPC (a), which function as control plasmids for examining the autoactivation of mRFP fragments. GOI were integrated as PCR-fragments digested with *Bam*HI/*Xho*I and ligated into *Bam*HI/*Sal*I sites in pCB:GOI-mRFPN and pCB:GOI-mRFPC (b) and pCB:mRFPN-GOI and pCB:mRFPC-GOI (c), respectively, yielding binary plasmids encoding mRFPN and mRFPC fusions that demonstrate the functionality of the system. pe35S = enhanced 35S promoter of CaMV, TL = translational enhancer of TEV, MCS = multiple cloning site (51 bp), GOI = gene of interest, myc = c-myc tag, HA = hemagglutinin tag, mRFPN (1-504) = N-terminal part of mRFP1-Q66T encoded by nucleotides 1-504, mRFPC (505-672) = C-terminal part of mRFP1-Q66T encoded by nucleotides 505-672, linker = amino acid linker, 35SpA = polyadenylation signal from CaMV.

In a subsequent step, plasmids pCB:mRFPN-GOI and pCB:mRFPC-GOI were generated by PCR mutagenesis. Plasmids pCB:PPV_CP-mRFPN and pCB:PPV_CP-mRFPC served as templates for PCR with the primer pairs 2453/2454 and 2454/2455, respectively, generating plasmids pCB:myc-mRFPN and pCB:HA-mRFPC, which contain the myc and HA tag, respectively. In a second PCR mutagenesis, the linker sequence and *Bam*HI/*Sal*I cloning sites were introduced downstream of mRFPN and mRFPC using primers 2456/2457 (Tab. 1) with the plasmid pCB:myc-mRFPN and primers 2456/2458 (Tab. 1) with the plasmid pCB:HA-mRFPC, resulting in pCB:mRFPN-*Bam*HI/*Sal*I and pCB:mRFPC-*Bam*HI/*Sal*I. GOI were PCR amplified, digested with *Bam*HI/*Xho*I and ligated into plasmids digested with *Bam*HI/*Sal*I to yield the final plant expression plasmids (Fig. 1c).

To establish an appropriate interacting and non-interacting control for BiFC, two PPV_CP deletion mutants were generated (Fig. 2a), replicating two CP deletion mutants of SMV-G7H described by Kang *et al.* (2006). For this purpose, full-length PPV_CP (PPV_CP_{full}) was shortened by PCR mediated mutagenesis. Fragment F1, encoding the N-terminal aa 1-97, and F3, encoding the C-terminal aa 222-315 of PPV_CP, were generated using pGEM®-T Easy_CP (pGEM®-T Easy; Promega, Madison, USA) as a template. The construct pGEM®-T Easy_F1 was generated by PCR amplification using primers PPV_N1 and PPV_N2 (Tab. 1), which delete the coding sequence for aa 98-315 of CP and introduce an *Xho*I-site. The construct pGEM®-T Easy_F3 was generated using primers PPV_C1 and PPV_CC2 (Tab. 1), which delete the coding sequence for aa 1-221 of the PPV_CP and introduce a *Bam*HI-site. Plasmids pGEM®-T Easy_F1 and pGEM®-T Easy_F3 were digested with *Bam*HI/*Xho*I and F1 and F3 were integrated into the pCB301-derived BiFC plasmids, digested with *Bam*HI/*Sal*I, yielding the respective plant expression plasmids.

Additionally, entire coding sequences of the N-protein of CaCV (Knierim *et al.*, 2006), CP of TMV and BC1 and BV1 of TYLCTHV (Blawid *et al.*, 2008) were PCR amplified using specific sense and antisense primers (Tab. 1) that introduce flanking *Bam*HI and *Xho*I sites. Fragments were subcloned into a modified pBluescript II KS(+) plasmid using *Bam*HI/*Xho*I restriction sites and subsequently introduced into pCB:PPV_CP-mRFPN and pCB:PPV_CP-mRFPC or pCB:mRFPN-GOI and pCB:mRFPC-GOI using *Bam*HI/*Sal*I restriction sites to replace PPV_CP and give the respective plasmids. All relevant inserts generated in this study were verified by sequencing (Sequence Laboratories, Göttingen, Germany).

2.3.2 Transient protein expression in *N. benthamiana* leaf epidermal cells

For transient protein expression in *N. benthamiana* leaf epidermal cells, binary plant expression plasmids were transferred to *A. tumefaciens* strain C58C1 by electroporation (Deblaere *et al.*, 1985). *A. tumefaciens* cultures harboring the BiFC plasmids and binary plasmid pCH32 encoding the p19 protein of TBSV (kindly provided by B. Kommor, University of Münster, Germany) were prepared for infiltration essentially as described by Voinnet *et al.* (2003). Optical density (600 nm) was measured and suspensions were adjusted with inoculation buffer (10 mM $MgCl_2$, 10 mM 2-[N-morpholino]

ethanesulfonic acid (MES), pH 5.6, 100 mM acetosyringone) to 2.0 ± 0.05 for agro-cultures harboring the BiFC-plasmids and 0.6 ± 0.05 for the agro-culture harboring the suppressor of gene silencing. For inoculation, the bacterial suspensions were mixed at a 1:1:2 (mRFPN:mRFPC:suppressor) ratio and agroinoculation was then performed by infiltrating the lower surface of the upper leaves of 4-5 week old *N. benthamiana* plants with *A. tumefaciens* mixtures using a 2 ml syringe lacking a needle. Plants were kept for three days at room temperature before evaluation by confocal laser scanning microscopy (CLSM).

2.3.3 Confocal laser scanning microscopy (CLSM)

For CLSM, small leaf discs (∅ 1 cm) from inoculated *N. benthamiana* leaves were cut and placed upside down on microscope slides. Interactions between proteins and protein fragments were analyzed using a Leica SP2 confocal microscope (Leica Microsystems, Wetzlar, Germany), operated by the Leica confocal software package. The mRFP was excited with the 543 nm line of the green neon laser (GreNe), and fluorescence emission was detected under constant record conditions in the red channel (600-610 nm).

2.3.4 Immunoblot analysis

Additionally, the expression of the protein fusions was verified in total leaf protein extracts. Single patches of inoculated leaves were analyzed by CLSM, and protein extracts of these leaves were prepared following the method of Berger *et al.* (1989). Protein samples were separated by Tricine/SDS-PAGE (Schägger & von Jagow, 1987) using 15% separation and 4% stacking polyacrylamide gels, and western blots were prepared from the gels, following the method of Towbin *et al.* (1979). Proteins were electroblotted onto nitrocellulose membranes (pore size 0.45 µm; Whatman, Maidstone, United Kingdom), which were incubated in blocking solution (1% blocking milk (Loewe Biochemica, Sauerlach, Germany) with 0.1% Tween 20 in phosphate-buffered saline (PBS) (137 mM NaCl, 1.47 mM KH_2PO_4, 7.81 mM Na_2HPO_4, 2.68 mM KCl, pH 7.4)). Membranes were probed overnight with primary anti-c-myc mouse monoclonal antibody (Roche Applied Science, Mannheim, Germany; 11667149001, 1:400) or anti-HA high affinity rat monoclonal antibody (Roche Applied Science,

Mannheim, Germany; 11867423001, 1:1000). After washing in PBS, membranes were probed with species-specific horseradish peroxidase (HRP)-conjugated goat anti-rat polyclonal IgG (Jackson ImmunoResearch Laboratories, Baltimore, USA; 112-036-003, 1:1000) or HRP-conjugated goat anti-mouse polyclonal IgG (Jackson ImmunoResearch Laboratories, Baltimore, USA; 115-035-003, 1:10,000), respectively, for 1 h. For detection of secondary antibodies by enhanced chemoluminescence, membranes were incubated in Luminol reaction mix (100 mM Tris, pH 8.5, 200 µM p-coumaric acid, 1.25 mM Luminol, 0.01% H_2O_2). Luminescence was subsequently detected with an Intas Chemoluminescence Imager (ChemoCam HR 16 3200; Intas Science Imaging Instruments, Göttingen, Germany) operated by Chemostar Software.

2.4 Results

An mRFP-based BiFC assay was performed to detect plant viral protein-protein interactions using two different sets of optimized plasmids. The first optimization step for this system was the integration of a 7-aa linker encoding sequence between the GOI and mRFP fragments. Self-interaction of two proteins, PPV_CP and *E. coli* GUS, was tested with the GGGSGGG-linker encoding plasmids, pBIN19:GOI-linker-mRFPN and -mRFPC, as well as constructs pBIN19:GOI-attR2-mRFPN and -mRFPC, which encode fusions with a linker sequence representing the residual attR2 of the Gateway®-Cloning system used by Jach *et al.* (2006) in the original system. Self-interaction of CPs of other potyviruses has already been demonstrated (Guo *et al.*, 2001; Kang *et al.*, 2004, 2006; Lin *et al.*, 2009; Shen *et al.*, 2010), and *E. coli* GUS is known to form tetramers (Bracey and Paigen, 1987). However, for PPV_CP self-interaction, no fluorescence was detected for the attR2-constructs (Fig. 2b), whereas co-expression of PPV_CP-linker-mRFPN and PPV_CP-linker-mRFPC clearly revealed fluorescence in epidermal cells (Fig. 2c). In the case of GUS self-interaction, only weak fluorescence was detected for the attR2 constructs (Fig. 2d). However, as for PPV_CP, strong red fluorescence in *N. benthamiana* leaves was obtained after agroinoculation with plasmid combinations encoding GUS protein fusions, separated by the amino acid linker sequence GGGSGGG, showing GUS self-interaction (Fig. 2e) and confirming an optimization of this system for analyzing protein-protein interactions *in planta*. Further studies were therefore only performed using the GGGSGGG-linker encoding constructs.

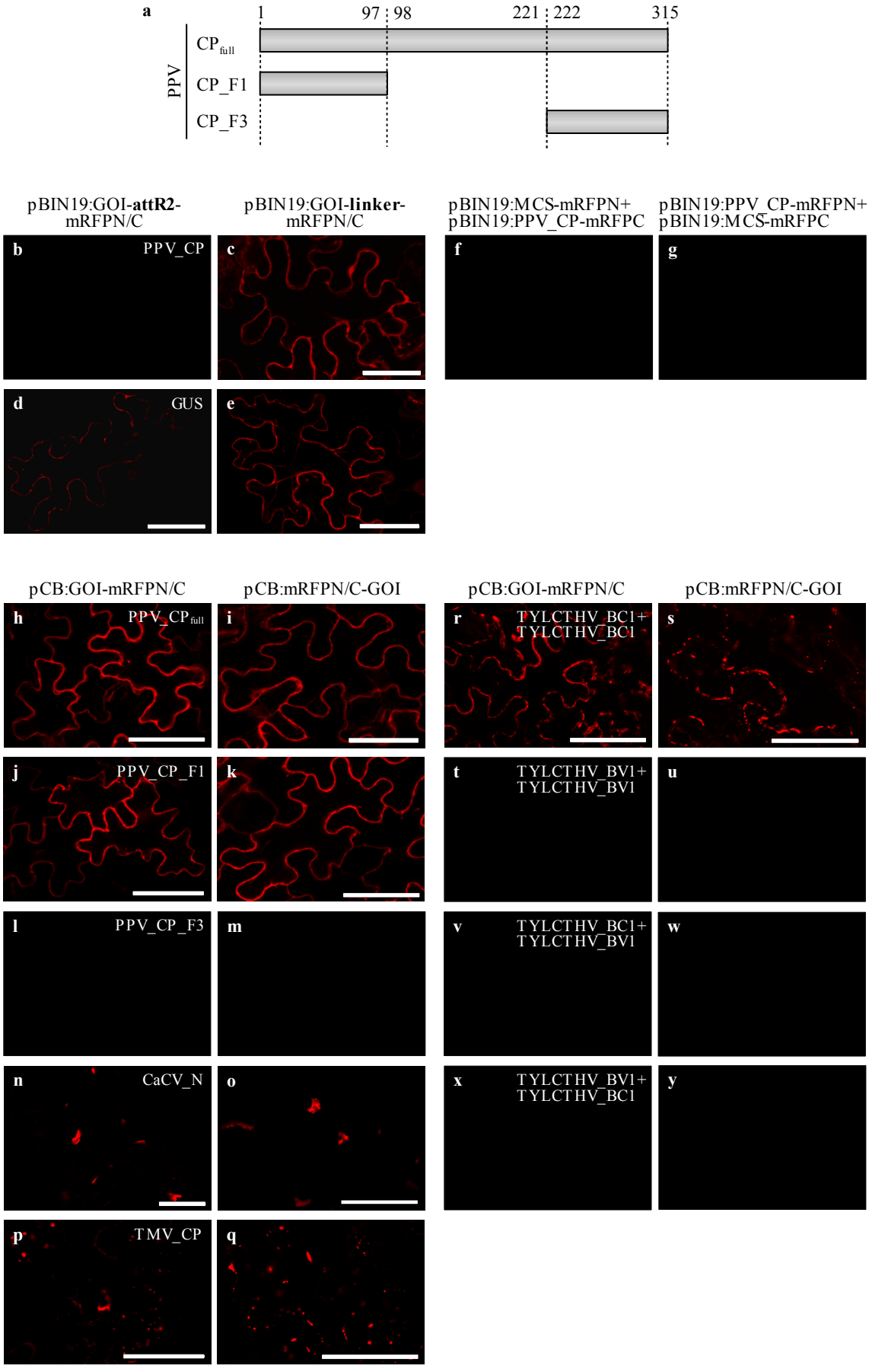

Fig. 2: Bimolecular fluorescence complementation of mRFP in *N. benthamiana* epidermal leaf cells.

(a) Map of the PPV_CP domains for the generation of an interacting and a non-interacting control for BiFC. Full-length CP (CP_{full}) was shortened by PCR. CP_{full} and fragments F1 and F3 contain aa 1-315, aa 1-97 and 222-315, respectively. (b-y) CLSM of epidermal leaf cells co-infiltrated with mixtures of agrobacteria harboring different BiFC expression plasmids. (b-e) Comparison of linker- and attR2-constructs. Cells were co-inoculated with plasmids pBIN19:PPV_CP-attr2-mRFPN and -mRFPC (b), pBIN19:PPV_CP-linker-mRFPN and -mRFPC (c), pBIN19:GUS-attR2-mRFPN and -mRFPC (d) and pBIN19:GUS-linker-mRFPN and -mRFPC (e). (f and g) No autoactivation of mRFP fragments was observed in cells co-inoculated with plasmids pBIN19:MCS-mRFPN and pBIN19:PPV_CP-mRFPC (f) and pBIN19:PPV_CP-mRFPN and pBIN19:MCS-mRFPC (g). (h-q) Test for homodimerization of different plant viral proteins (indicated on the left side of each picture) by co-inoculation of cells using plant expression plasmids pCB:GOI-mRFPN and -mRFPC (h, j, l, n, p) and pCB:mRFPN-GOI and pCB:mRFPC-GOI (i, k, m, o, q). (r-y) Test for self-interaction and "cross-interaction", respectively, of BC1 and BV1 of TYLCTHV by co-inoculation of cells using plant expression plasmids pCB:GOI-mRFPN and -mRFPC (r, t, v, x) and pCB:mRFPN-GOI and pCB:mRFPC-GOI (s, u, w, y). Scale bars: 50 μm.

In a further step, autoactivation of mRFP fragments was tested by performing experiments using either mRFPN or mRFPC fragments that contained only a short peptide sequence encoded by the MCS and no fusion partner. Co-expression of MCS-mRFPN and PPV_CP-mRFPC, as well as PPV_CP-mRFPN and MCS-mRFPC, revealed no fluorescence (Fig. 2f-g), demonstrating that there is no autoactivation of mRFP fragments.

To demonstrate the functionality of the modified BiFC system, proteins of interest, PPV_CP and deletion mutants F1 and F3 thereof, CaCV N-protein and TMV_CP, were tested for self-interaction using the plasmid set pCB:GOI-mRFPN and pCB:GOI-mRFPC, which encode N-terminal mRFP-fusions and the plasmid set pCB:mRFPN-GOI and pCB:mRFPC-GOI, encoding C-terminal mRFP fusions. To validate the BiFC system further, BC1 and BV1 of TYLCTHV were also tested with both plasmid sets, examining both self-interaction and 'cross-interaction' of the two proteins. Fluorescence was subsequently observed 3 days past inoculation (dpi) by CLSM, and Fig. 2h-y shows the results of these experiments. When PPV_CP_{full}-mRFP and mRFP-PPV_CP_{full} fragments were co-expressed, red fluorescence, resulting from reconstitution of functional mRFP, was observed in the cytoplasm of epidermal cells, exposing the outlines of the epidermal cells (Fig. 2h-i). This result indicates the self-interaction of PPV_CP_{full}. Similar results were obtained for PPV_CP_F1 with both plasmid sets (Fig. 2j-k). In the case of PPV_CP_F3, no fluorescence was detected in the epidermal cells with either plasmid set (Fig. 2l-m), indicating that the C-terminal part of PPV_CP, consisting of aa 222-315, is not involved in self-interaction of the protein.

Co-inoculation of the CaCV_N-mRFP constructs resulted in strong mRFP signals located in small aggregates all over the cytoplasm (Fig. 2n-o), indicating the self-

interaction of this protein, as expected. Using TMV_CP, strong red fluorescence was observed in little aggregates along the cell wall (Fig. 2p-q), demonstrating a TMV_CP self-interaction, which is consistent with aspects of TMV particle assembly (Bendahmane *et al.*, 1999; Namba *et al.*, 1989). To validate the BiFC system further, BC1-mRFP constructs and BV1-mRFP constructs were co-expressed in epidermal leaf cells. With BC1, red fluorescence in little aggregates localized along the cell wall was detected (Fig. 2r-s). In the case of BV1, neither co-expression of the N-terminal mRFP fusions nor the C-terminal mRFP fusions revealed fluorescence (Fig. 2t-u). Furthermore, no fluorescence was found in plant cells when co-expression of BV1-mRFPN and BC1-mRFPC and *vice versa* combinations were tested (Fig. 2v-y). The results of these experiments with BC1 and BV1 of TYLCTHV are in complete agreement with the results of Frischmuth *et al.* (2004) concerning BC1 and BV1 of AbMV.

To confirm that the lack of fluorescence in the BiFC assay is due to the absence of protein-protein interaction, and not from the loss of expression of the fusion proteins, immunoblot analyses with specific anti-c-myc and anti-HA antibodies were performed. Immunodetection of transiently co-expressed mRFPN and mRFPC fusion proteins in plant leaves revealed that all fusion proteins were expressed in inoculated leaves.

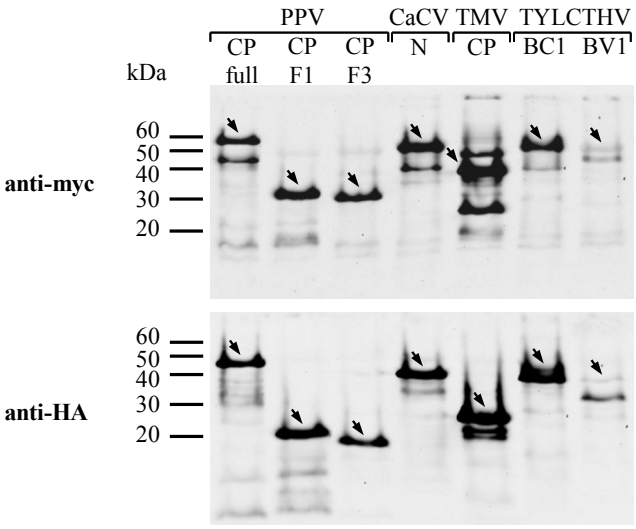

Fig. 3: Immunodetection of transiently co-expressed mRFPN and mRFPC fusion proteins in *N. benthamiana* leaves. mRFPN fusion proteins were identified with anti-c-myc and mRFPC fusion proteins with anti-HA monoclonal antibodies in immunoblot analyses of *N. benthamiana* leaf material 3 dpi. Fusion proteins analyzed are stated above each lane. Arrows show full-length fusion proteins at their calculated sizes. A molecular mass ladder is indicated at the left.

The estimated molecular weights were in accordance with the calculated molecular weights; the respective bands are marked with arrowheads in Fig 3. Linker-myc-mRFPN and linker-HA-mRFPC sequences caused a shift of approximately 20.5 and 8.5 kDa, respectively, in the fusion proteins. BV1 protein fusions were expressed at significantly lower levels in the plant cells compared to BC1 or other viral protein fusions. However, this is in accordance with results from immunoblot analyses in yeast cells by Frischmuth *et al.* (2004) where BV1 also accumulated in lower amounts than BC1. This may result from a high instability of the protein or faster degradation during protein extraction. Furthermore, for some proteins, more than one band was detected, possibly also due to the degradation of the fusion proteins during protein extraction or non-specific binding of the anti-c-myc antibody.

2.5 Discussion

An optimized mRFP-based BiFC system to detect protein-protein interactions *in planta* was developed. Two different plasmid sets were generated that encode different mRFP fusions. The two plasmid sets were used to analyze whether the order of the GOI and the mRFP, either as N- or C-terminal fusions, is crucial in detecting protein interactions. One plasmid set allows the GOI to be inserted upstream of the coding sequence for the mRFP fragment, thus expressing an N-terminal mRFP fusion; in the other case, the GOI is located downstream of the mRFP fragment, resulting in a C-terminal mRFP fusion protein. Kerppola (2006) describes the importance of testing both localizations of the GOI to ensure that the fluorescent protein parts are able to associate with each other upon interaction of the tested proteins. The analysis revealed no negative effect on the tested interactions for the modified BiFC system with respect to the position of the GOI relative to the mRFP. Therefore, the system enables the analysis of N- as well as C-terminal mRFP fusions with the protein of interest, representing an additional improvement over the original mRFP1-Q66T system.

Two major improvements were introduced into the existing BiFC system. Firstly, the most important feature of the optimized plasmids in the BiFC system, when compared to the original plasmid set, is the 7-aa linker (Schwartz *et al.*, 2004) encoding sequence introduced between the mRFP fragments and the GOI. Kerppola (2006) describes the

usage of such a linker as important for the maximal flexibility of the single components during complex formation. In previous studies, different linker sequences ranging between 5 and 17 aa in length were used successfully in different BiFC analyses. Bracha-Drori *et al.* (2004) also reports a 17-aa encoding linker sequence introduced between the single YFP fragments and GOI in plasmids for a BiFC in plants. In addition, a 10-aa linker was used by Fan *et al.* (2008) in the mCherry BiFC. In contrast to the GGGSGGG-linker in this work, a second linker representing the rudimentary attachment-site (SAFLYKVV) of the Gateway®-Cloning system was tested, but fusions revealed no or only weak interaction. The best signal intensity was clearly achieved with the GGGSGGG-linker, leading to an improvement over the original system. Secondly, a further modification concerns the vector system used for agroinoculation. In this study, pBIN19 was originally used as the vector backbone for plant expression plasmids. In further steps of optimization, a modified mini binary vector pCB301 (Xiang *et al.*, 1999) was introduced into the system, which is significantly smaller (~ 3.5 kb) than other binary vectors, e.g., pBIN19 (~ 12 kb; Bevan, 1984). This smaller vector has different advantages compared with larger ones, such as easy manipulation in *E. coli* and high stability (Hajdukiewicz *et al.*, 1994; Xiang *et al.*, 1999). Furthermore, the usage of the new vectors avoids expensive Gateway®-cloning, which is especially suitable for high throughput experiments.

Every new or modified system requires reliable controls, e.g., proteins that are known to interact, that can serve as positive controls. Since the original aim of this study was to develop a system that could detect plant viral protein-protein interactions, it was preferable to use plant viral proteins as controls. The CPs of some potyviruses have been shown to self-interact in yeast two-hybrid assays (Guo *et al.*, 2001; Kang *et al.*, 2004, 2006; Lin *et al.*, 2009; Shen *et al.*, 2010), and for this reason, PPV_CP served as a positive control. Moreover, the N-protein of CaCV, for which a self-interaction was shown for the first time, and TMV_CP were used to validate the system. Fluorescent protein fragments can form fluorescent complexes, albeit with a low efficiency, in the absence of specific protein interactions, and therefore, it was also necessary to develop a negative control (Kerppola, 2006). Proteins known not to interact are useful; however, better negative controls are proteins in which the interaction domain has been mutated or deleted and have been fused to the fluorescent fragment in a manner identical to the wild-type protein. This was implemented with PPV_CP. The F1 mutant, consisting of

the N-terminal aa 1-97 of the coat protein, was shown to self-interact, whereas the C-terminal 93 amino acids, representing the F3 mutant, showed no fluorescence in the BiFC assay and was thus revealed in this study to be unnecessary for the self-interaction of the PPV_CP. Therefore, F1 can be used as an additional interacting control and F3 as reliable non-interacting control in future studies. Surprisingly, these findings are in contrast to the yeast two-hybrid results for SMV-G7H_CP shown by Kang *et al.* (2006) where the F3 fragment of the CP was found to be necessary and sufficient for self-interaction. These inconsistent results indicate that the domains important for potyviral CP self-interaction are possibly not conserved among potyviruses. Finally, BV1 and BC1 served as additional controls to validate the system by reproducing the results shown for AbMV movement proteins (Frischmuth *et al.*, 2004).

Red fluorescence indicates interactions of the proteins of interest, and the experimental results for CP, N, BC1 and BV1 correspond with the expectations. The analyses revealed strong fluorescence of interacting protein complexes using the GGGSGGG-linker constructs in comparison to the attR2-constructs and were easy to reproduce. Neither false positive nor false negative interactions were detected under the indicated experimental conditions. The mini binary vectors ensure easy construction of expression vectors, and the linker increases the flexibility of the single fusion partners during the formation of the interaction complex. The optimized BiFC system provides a reliable and simple assay for the visualization of protein interactions in living cells in a natural physiological environment, meaning proteins are expressed in a biologically relevant context (Kerppola, 2006). *N. benthamiana* is an optimal system to test, e.g., plant viral interactions. Plants can be quickly transfected, and epidermal leaf cells are easily accessible for microscopic observation. A great advantage of BiFC analysis is the intrinsic fluorescence of the complex. This enables detection of protein interactions without the use of other exogenous reagents (Hu *et al.*, 2002). Moreover, the BiFC assay does not require specialized and expensive equipment. The BiFC assay is easier and much faster to handle than a yeast system and circumvents a couple of critical points. Nevertheless, one disadvantage of the BiFC may be its unsuitability for the screening of complete libraries. While single protein interactions can easily be analyzed with BiFC, library-screens should be performed in yeast two-hybrid systems.

In summary, this paper describes an optimized BiFC method with sets of plasmids providing fast and simple tools to detect single protein-protein interactions directly *in*

planta. This protocol provides a convenient alternative to other BiFC assays, as well as other methods used to detect protein-protein interactions, like FRET or yeast two-hybrid systems, which enable the detection of protein interactions *in vivo* but only with highly technical or relatively laborious efforts, respectively.

3 Detection of plum pox potyviral protein-protein interactions *in planta* using an optimized mRFP-based bimolecular fluorescence complementation system

3.1 Abstract

In previous studies, protein interaction maps of different potyviruses have been generated using yeast two-hybrid (YTH) systems, and these maps have demonstrated a high diversity of interactions of potyviral proteins. Using an optimized bimolecular fluorescence complementation (BiFC) system, a complete interaction matrix for proteins of a potyvirus was developed for the first time under *in planta* conditions with ten proteins from *Plum pox virus* (PPV). In total, 52 of 100 possible interactions were detected, including the self-interactions of CI, 6K2, VPg, NIa-Pro, NIb and CP, which is more interactions than have ever been detected for any other potyvirus in a YTH approach. Moreover, the BiFC system was shown to be able to localize the protein interactions, which was typified for the protein self-interactions indicated above. Additionally, experiments were carried out with the P3N-PIPO protein, revealing an interaction with CI but not with CP and supporting the involvement of P3N-PIPO in the cell-to-cell movement of potyviruses. No self-interaction of the PPV helper component-proteinase (HC-Pro) was detected using BiFC *in planta*. Therefore, additional experiments with *Turnip mosaic virus* (TuMV) HC-Pro, PPV_HC-Pro and their mutants were conducted. The self-interaction of TuMV_HCpro, as recently demonstrated, and the self-interaction of the TuMV_ and PPV_HC-Pro mutants were shown by BiFC *in planta*, indicating that HC-Pro self-interactions may be species-specific. BiFC is a very useful and reliable method for the detection and localization of protein interactions *in planta*, thus enabling investigations under more natural conditions than studies in yeast cells.

Keywords: *Plum pox virus* (PPV), protein-protein interaction, bimolecular fluorescence complementation (BiFC), yeast two-hybrid system, P3N-PIPO, helpercomponent-proteinase (HC-Pro)

3.2 Introduction

Plum pox virus (PPV) is a member of the genus *Potyvirus* within the family *Potyviridae*, which includes approximately 30 % of all known plant viruses. PPV and other members of the genus, like *Soybean mosaic virus* (SMV) and *Turnip mosaic virus* (TuMV), are of horti- and agricultural importance because they cause significant losses in a wide range of plants. Potyviruses are flexible filaments with a single-stranded, positive-sense RNA genome of approximately 10 kb. At its 5`-end the RNA is linked covalently to the VPg (viral genome-linked protein), and the 3`-end carries a poly(A) tail. The RNA comprises one long open reading frame (ORF) that is translated into a polyprotein precursor of approximately 350 kDa. This precursor is processed by three virus-encoded proteinases, which release ten mature viral proteins: the P1 protein; helpercomponent-proteinase (HC-Pro); P3 protein; a first peptide of 6 kDa (6K1); cylindrical inclusion (CI) protein; a second peptide of 6 kDa (6K2); nuclear inclusion protein a (NIa), with the N-terminal VPg and a C-terminal proteinase (NIa-Pro); nuclear inclusion protein b (NIb); and coat protein (CP) (Riechmann *et al.*, 1992; Shukla *et al.*, 1994; López-Moya *et al.*, 2000). Recently, the discovery of a further short ORF, called PIPO (pretty interesting *Potyviridae* ORF), has been reported for potyviruses (Chung *et al.*, 2008; Wen & Hajimorad, 2010; Wei *et al.*, 2010a). This short ORF is embedded within the P3 cistron and translated by a frameshift in the +2 reading frame. The encoded protein is a fusion with the N-terminal part of P3, giving rise to P3N-PIPO.

Most potyviral proteins are multifunctional and participate in different phases of the virus infection cycle, e.g., in aphid-transmission (Blanc *et al.*, 1997, 1998; Plisson *et al.*, 2003), virus replication, and cell-to-cell (Carrington *et al.*, 1998) or long-distance (Cronin *et al.*, 1995; Kasschau & Carrington, 2001) movement. An overview on potyviral protein functions has been given previously by Urcuqui-Inchima *et al.* (2001) and Rajamäki *et al.* (2004). The identification and investigation of protein-protein interactions comprise an important step in understanding cellular processes, the viral infection cycle and the interplay between virus and host. Several methods have been developed over the last decades to identify and examine protein-protein interactions. In addition to different *in vitro* methods (Phizicky & Fields, 1995), the yeast two-hybrid (YTH) system (Fields & Song, 1989) is the most popular *in vivo* method for the detection of protein interactions. The method is based on the transcriptional activation of a reporter gene in the yeast nucleus after the interaction of two proteins of interest,

which are fused to the binding domain and activating region, respectively, of a transcription factor (Fields & Song, 1989). However, this system bears limitations, including systematic false negative and false positive interactions and the requirement that interacting proteins must accumulate in the yeast nucleus (Golemis *et al.*, 1999). Furthermore, even though the method gives the possibility to detect physical interactions, it does not represent interactions under natural conditions, and neither does it reflect biologically relevant modifications or subcellular localization of the proteins (Stolpe *et al.*, 2005).

Several interactions between potyviral proteins have been analyzed using different YTH systems (Hong et al, 1995; Li *et al.*, 1997; Guo *et al.*, 1999; Merits *et al.*, 1999; Urcuqui-Inchima *et al.*, 1999; Choi *et al.*, 2000; López *et al.*, 2001; Roudet-Tavert *et al.*, 2002; Yambao *et al.*, 2003; Seo *et al.*, 2010). Moreover, complete interaction maps of potyviral proteins, excluding P3N-PIPO, of *Potato virus A* (PVA) and *Pea seed-borne mosaic virus* (PSbMV) (Guo *et al.*, 2001), *Papaya ringspot virus* strain P (PRSV-P) (Shen *et al.*, 2010), *Shallot yellow stripe virus* strain O (SYSV-O) (Lin *et al.*, 2009) and different strains of SMV, SMV-G7H and SMV-P (Kang *et al.*, 2004; Lin *et al.*, 2009), have been generated. So far, no complete interaction map of PPV proteins has been developed. However, López *et al.* (2001) analyzed the CI with respect to self-interaction and interaction with five other PPV proteins, giving a first, but incomplete, insight into the interactome of this virus.

Focusing on complete protein interaction maps within members of the genus *Potyvirus*, experiments with different viruses have not given consistent results for interactions between homologous viral proteins and do not necessarily correspond to the results from *in vitro* approaches (Merits *et al.*, 1999; López *et al.*, 2001). The diversity of interactions seems to be very high, and it is difficult to verify whether this has resulted from methodological limitations or inconsistent protein interaction behavior of the different potyviruses. The results obtained surely may serve as the basis for further studies, but subsequent examination with an *in vivo* method under natural conditions is useful and desirable.

Bimolecular fluorescence complementation analysis (BiFC) represents a powerful alternative approach to YTH assays for the study of protein-protein interactions in living cells. It was first developed to detect protein-protein interactions in mammalian tissue culture cells (Hu *et al.*, 2002; Hu & Kerppola, 2003), but was adapted meanwhile for

bacteria (Atmakuri *et al.*, 2003), yeast (Blondel *et al.*, 2005; Cole *et al.*, 2007; Sung & Huh, 2007) and plant systems (Walter *et al.*, 2004; Bracha-Drori *et al.*, 2004; Citovsky *et al.*, 2008). The method is based on the formation of a fluorescent complex when the N- and C-terminal non-fluorescent halves of a fluorescent protein are brought together by the association of interaction partners fused to the protein fragments (Hu *et al.*, 2002). The method enables a fast and direct real-time visualization of the protein complex under natural conditions, which is the main advantage over other methods, especially over the YTH system.

In the present study, experiments were carried out to detect protein-protein interactions of the PPV proteins in *Nicotiana benthamiana* cells. A recently optimized mRFP-based BiFC system (Zilian & Maiss, 2011) was used to generate a complete interaction map of ten proteins from PPV, representing the first BiFC-based potyviral interaction map. In an initial step PPV_CP and different mutants were used to develop interacting and non-interacting controls. Further experiments focused on the interaction of P3N-PIPO and CI, which was expected to be important for cell-to-cell movement of the virus (Wei *et al.*, 2010b). Moreover, it is demonstrated that, in addition to the detection of protein interactions, the system offers the capability to visualize the localization of interacting proteins, which has been reported previously (Citovsky *et al.*, 2006, 2008; Martin *et al.*, 2009).

3.3 Materials and methods

3.3.1 Construction of the expression plasmids for BiFC

The pBIN19:GOI-mRFPN and -mRFPC, pCB:GOI-mRFPN and -mRFPC and pCB:mRFPN-GOI and pCB:mRFPC-GOI expression plasmids were generated as described previously (Zilian & Maiss, 2011; Fig. 1).

3.3.2 Construction of the PPV_CP plasmids

Full-length CP, derived from a PPV-NAT full-length clone (Maiss *et al.,* 1992), was PCR amplified with primers CPY2H_s and CPY2H_as (Tab. 1) and introduced into pGEM®-T Easy (Promega, Madison, USA) giving pGEM®-T Easy_CP. The CP gene

was divided into five fragments by PCR mutagenesis using Phusion Flash DNA polymerase (Finnzymes, Espoo, Finland). The F1, F3, F1_2 and F2_3 fragments of PPV_CP, encoding aa 1-97, aa 222-315, aa 1-221 and aa 98-315, respectively, were generated using pGEM®-T Easy_CP as the template. The plasmids pGEM®-T Easy_F1, pGEM®-T Easy_F3, pGEM®-T Easy_F1_2 and pGEM®-T Easy_F2_3 were PCR-amplified using the PPV_N1/PPV_N2, PPV_C1/PPV_CC2, PPV_N1/PPV_NC2 and PPV_CC1/PPV_CC2 primer pairs (Tab. 1), respectively. Plasmid pGEM®-T Easy_F2, encoding aa 98-221 of PPV_CP, was generated by PCR amplification of pGEM®-T Easy_F2_3 with the PPV_N1/PPV_NC2 primer combination (Tab. 1). Finally, CP_{full} and CP fragments F1, F1_2, F2, F2_3 and F3 were *Bam*HI/*Xho*I-digested and ligated into binary plasmids digested with *Bam*HI/*Sa*lI.

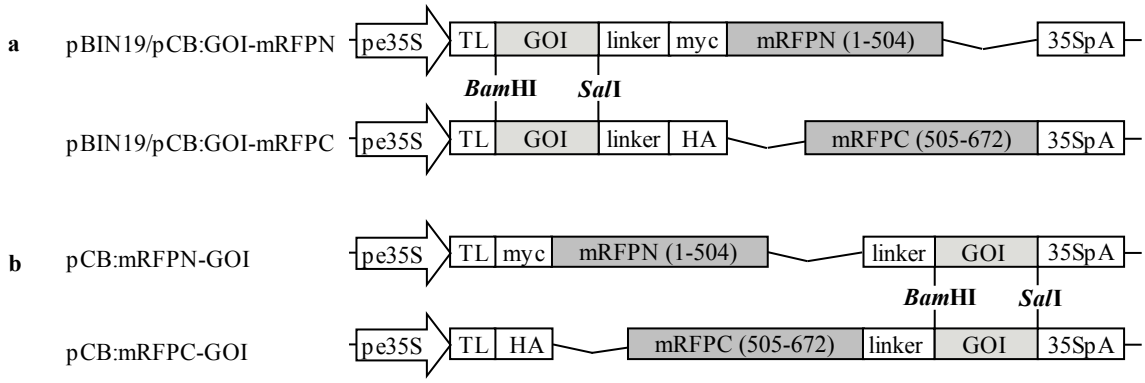

Fig. 1: Schematic representation of the expression cassettes of optimized BiFC plasmids. PCR fragments of the genes of interest (GOI) were integrated by digestion with *Bam*HI/*Xho*I (except for NIa-pro: *Bgl*II/*Sal*I) and insertion into the *Bam*HI and *Sal*I sites of the pBIN19:GOI-mRFPN and -mRFPC or pCB:GOI-mRFPN and -mRFPC binary plasmids, encoding the N-terminal mRFP fusions (a) and the pCB:mRFPN-GOI and pCB:mRFPC-GOI plasmids , which encode the C-terminal mRFP fusions (b). pe35S = enhanced 35S promoter of CaMV, TL = translational enhancer of TEV, myc = c-myc tag, HA = hemagglutinin tag, mRFPN (1-504) = N-terminal part of mRFP1-Q66T encoded by nucleotides 1-504, mRFPC (505-672) = C-terminal part of mRFP1-Q66T encoded by nucleotides 505-672, linker = amino acid linker, 35SpA = polyadenylation signal of CaMV.

3.3.3 Construction of the plasmids for PPV full-length protein interactions

The PPV genes P1, HC-Pro, P3, 6K1, CI, 6K2, VPg, NIa-Pro and NIb were PCR-amplified from the PPV-NAT full-length clone (Maiss *et al.*, 1992) using Phusion Flash DNA polymerase (Finnzymes) and specific sense and antisense primers (Tab. 1) that introduced flanking *Bam*HI and *Xho*I sites (except for NIa-Pro: *Bgl*II/*Sal*I). The PCR-amplified fragments were digested with *Bam*HI/*Xho*I (except for NIa-Pro: *Bgl*II/*Sal*I) and inserted into binary plasmids digested with *Bam*HI/*Sal*I (except for NIa-Pro: *Bgl*II/*Sal*I), yielding the respective BiFC plasmids.

Tab. 1: Oligonucleotides

Fragment	Primer	Sequence (5` → 3`) [a]	Restriction sites
Amplification of PPV_CP$_{full}$ and construction of PPV_CP mutants.			
CP$_{full}$	CPY2H_s	AAAGGATCCGCTGACGAAAGAGAAGACGAG	*Bam*HI
	CPY2H_as	TTTCTCGAGCACTCCCCTCATACCGAGG	*Xho*I
	PPV_N1	CTCGAGAAAAATCACTAGTGAATTCG	*Xho*I
	PPV_N2	TGCCTTCAAACGTGGCACTGTA	
	PPV_C1	CGAAATTATGAAAAAGCATACATGC	
	PPV_CC2	GGATCCTTTAATCGAATTCCCG	*Bam*HI
	PPV_NC2	TTTTTCAATATACGCTTCAGCC	
	PPV_CC1	ATGACTTCGAAACTA TCT CTG CC	
Amplification of PPV full-length genes and mutagenesis of P3N-PIPO plasmids.			
P1	P1Y2H_s	AAAGGATCCATGTCAACCATTGTATTTG	*Bam*HI
	P1Y2H_as	TTTCTCGAGGTAGTGGATTATCTCATTG	*Xho*I
HC-Pro	HC-ProY2H_s	AAAGGATCCTCAGACCCAGGCAAACAAT	*Bam*HI
	HC-ProY2H_as	TTTCTCGAGTCCAACCAGGTATGTTTTC	*Xho*I
P3	P3Y2H_s	AAAGGATCCGGTCTTGAAGTGGATAAGTG	*Bam*HI
	P3Y2H as	AAACTCGAGTTGATGAACAACAACTTGAC	*Xho*I
6K1	6K1Y2H_s	AAAGGATCCAGTAAGAGAGACTCACAAGC	*Bam*HI
	6K1Y2H_as	AAACTCGAGCTGATGATGAACAGCCCGGT	*Xho*I
CI	CIY2H_s	AAAGGATCCAGCTTGGACGATATAGAAGA	*Bam*HI
	CIY2H_as	AAACTCGAGTTGATGGTGCACACATTCTA	*Xho*I
6K2	6K2Y2H_s	AAAGGATCCACAAAGGAAGGAGTTTCAAA	*Bam*HI
	6K2Y2H_as	AAACTCGAGTTGGTGAATAACTTCTTCTT	*Xho*I
VPg	VPgY2H_s	AAAGGATCCGGTTTCAATCGTAGGCAAAG	*Bam*HI
	VPgY2H_as	AAACTCGAGTTCGTGGTCAACTTCTTCGT	*Xho*I
NIa-Pro	NIa-Pro_s	AAAAGATCTAGTAAATCACTGTTTAGAGGCC	*Bgl*II
	NIa-Pro_as	AAAGTCGACCTGAGTGTAAACAAATTCCC	*Sal*I
NIb	NIbY2H_s	AAAGGATCCTCCAAAACTACACATTGGCT	*Bam*HI
	NIbY2H_as	AAACTCGAGTTGGTGCACAACAACGTTGG	*Xho*I
P3N-PIPO	PIPO_s	AAAAAAAGTTATCTCCAGGAATTGGAGCAAG	
	PIPO_as	CTACCAAGTGGGGTGTTGCCTGTCTGAG	
	PIPO_*Xho*I_as	AAACTCGAGGGAAGAAAACTTGGT	*Xho*I
Mutagenesis of TuMV_HC-Pro and PPV_HC-Pro.			
TuMV_HC-Pro	TuMV_HC-Pro_s	AAAGGATCCAGTGCAGCAGGAGCCAAC	*Bam*HI
	TuMV_HC-Pro_as	AAAGTCGACTCCAACGCGGTAGTGTTT	*Sal*I
	PPV_HC-Pro_N1	TAGAAAAGCGAGAACATGTG	
	PPV_HC-Pro_N2	GGAGGTGGATCTGGTGGAGGTA	
	PPV_HC-Pro_C1	GGATCCCATTGTTATATCTCCTTCG	
	PPV_HC-Pro_C2	AAGAGATATCGTGAACTAATGCGCG	
	TuMV_HC-Pro_N1	CAGTATTTGCACTGCGTGTTTGAATC	
	TuMV_HC-Pro_C2	GACAGGTATGAACACTCACTCAGTAG	

[a] Restriction sites are underlined.

3.3.4 Construction of the PPV_P3N-PIPO plasmids

The plasmids pCB:mRFPN-P3 and pCB:mRFPC-P3 served as templates for the generation of the PPV_P3N-PIPO-encoding plasmids pCB:mRFPN-P3N-PIPO and pCB:mRFPC-P3N-PIPO. PCR mutagenesis was performed using Phusion Flash Master Mix (Finnzymes) and the primers Pipo_s and Pipo_as (Tab. 1), which introduced a +2 frameshift into the P3 cistron.

To generate pCB:P3N-PIPO-mRFPN and pCB:P3N-PIPO-mRFPC, the P3N-PIPO gene was PCR-amplified from pCB:mRFPN-P3N-PIPO using the P3Y2H_s and PIPO_*Xho*I_as primers (Tab. 1), which introduce *Bam*HI and *Xho*I sites and delete the stop codon. The P3N-PIPO PCR fragment was digested with *Bam*HI/*Xho*I and integrated into pCB:GOI-mRFPN and -mRFPC. All inserts were verified by sequencing (Sequence Laboratories, Göttingen, Germany).

3.3.5 Construction of the TuMV_HC-Pro and PPV_HC-Pro plasmids

The coding sequence of TuMV_HC-Pro was RT-PCR-amplified using a total RNA preparation from a TuMV [DSMZ PV-0104]-infected *N. benthamiana* plant with RevertAid Premium Reverse Transcriptase (Thermo Fisher, St. Leon-Rot, Germany) and Phusion Flash Master Mix using the TuMV_HCpro_s and TuMV_HCpro_as primers (Tab. 1), which introduced flanking *Bam*HI and *Sal*I sites. The generated fragment was digested with *Bam*HI/*Sal*I and used to replace PPV_CP in pCB:CP-mRFPN and pCB:CP-mRFPC. The resulting plasmids were designated pCB:TuMV_HC-Pro-mRFPN and pCB:TuMV_HC-Pro-mRFPC.

These two plasmids and pCB:PPV_HC-Pro-mRFPN and pCB:PPV_HC-Pro-mRFPC were used as templates for the PCR mutagenesis of the TuMV_HC-Pro and the PPV_HC-Pro constructs. Plasmids encoding the mRFP fusions with the N-terminal parts of the HC-Pros (aa 1-99) were generated by PCR mutagenesis with Phusion Flash Master Mix using PPV_HC-Pro_N1 and HC-Pro_N2 with PPV_HC-Pro and primers TuMV_HC-Pro_N1 and HC-Pro_N2 (Tab. 1) with TuMV_HC-Pro. The resulting plasmids were designated pCB:PPV_HC-ProN-mRFPN and -mRFPC and pCB:TuMV_HC-ProN-mRFPN and -mRFPC. For the generation of plasmids encoding mRFP fusions with the C-terminal regions of the HC-Pros (aa 100-458), PCR

mutagenesis was performed using the primers HC-Pro_C1 and PPV_HC-Pro_C2 with PPV_HC-Pro and primers HC-Pro_C1 and TuMV_HC-Pro_C2 (Tab. 1) with TuMV_ HC-Pro, to yield the pCB:PPV_HC-ProC-mRFPN and -mRFPC and pCB:TuMV_HC-ProC-mRFPN and -mRFPC plasmids.

3.3.6 Transient protein expression in *N. benthamiana* leaf epidermal cells and CLSM

Binary plant expression plasmids were electroporated into *Agrobacterium tumefaciens* strain C58C1 (Deblaere *et al.*, 1985) for the infiltration of *N. benthamiana* plants. Agrobacterial cultures harboring the BiFC plasmids and pCH32 binary plasmid, encoding the p19 protein of TBSV, were prepared for infiltration as described by Zilian & Maiss (2011). Young leaves of 4-5-week-old *N. benthamiana* plants were infiltrated with mixtures of *A. tumefaciens*. The plants were incubated for three days under room temperature before single discs of inoculated *N. benthamiana* leaves were assayed for fluorescence by CLSM using a Leica TCS SP2 confocal microscope (Leica Microsystems, Wetzlar, Germany). The mRFP was excited at 543 nm with the green neon laser (GreNe) and the emitted light was captured at 600 to 610 nm under constant record conditions. The images were captured digitally and processed using the Leica confocal software.

3.3.7 Immunoblot analysis

Expression of the protein fusions was verified in total leaf protein extracts using anti-HA- and anti-c-myc-specific monoclonal antibodies for detection. Protein extracts at 3 dpi were prepared from leaves expressing GOI-mRFPN and GOI-mRFPC fusions [modified after Berger *et al.* (1989)]. Protein samples were separated by Tricine/SDS-PAGE (Schägger & von Jagow, 1987) using 15 % separation and 4 % stacking polyacrylamide gels and western blots were prepared from the gels as previously described (Zilian & Maiss, 2011). The detection of luminescence was performed with an Intas Chemoluminescence Imager (ChemoCam HR 16 3200; Intas Science Imaging Instruments, Göttingen, Germany) operated by Chemostar Software.

3.4 Results and discussion

3.4.1 Identification of PPV_CP domains involved in CP self-interaction

An optimized BiFC system (Zilian & Maiss, 2011; Fig. 1) with mRFP as a reporter was used to analyze the interactions of PPV proteins. The binary plasmid pBIN19 (Bevan, 1984) and a modified mini binary plasmid, pCB301 (Xiang *et al.*, 1999), were used as the backbones for optimized BiFC plasmids, whereby the interactions between the proteins did not depend on a single plasmid backbone (data not shown).

PPV_CP dimer formation was tested in an initial analysis to validate the BiFC system, as the PPV_CP self-interaction was expected to be necessary for the encapsulation of the viral RNA. In addition, five different PPV_CP deletion mutants were generated and interactions between these mutants were tested. According to Kang *et al.* (2006) PPV_CP was divided into three domains yielding five mutants: F1, F1_2, F2_3, F2 and F3, consisting of aa 1-97, aa 1-221, aa 98-315, 98-221 and aa 222-315, respectively (Fig. 2a). To test the capability of the full-length CP (CP_{full}) and the mutants to interact with themselves and each other, *N. benthamiana* plants were inoculated with mixtures of three *A. tumefaciens* cultures harboring the expression plasmids pCB:GOI-mRFPN, pCB:GOI-mRFPC and pCH32. Plasmid pCH32 (kindly provided by B. Kommor, University of Münster, Germany), encoding the p19 protein of *Tomato bushy stunt virus* (TBSV), was used to suppress gene silencing (Voinnet *et al.,* 1999, 2003). Fluorescence was observed 3 days past inoculation (dpi) by CLSM (Fig. 2b-e).

Co-expression of CP_{full}-mRFP, F1-mRFP and F2-mRFP fragments revealed red fluorescence in the cytoplasm of epidermal cells (Fig. 2b-d), representing self-interaction of CP_{full}, F1 and F2 mutants, respectively. However, after co-inoculation of the F3-mRFP fragments, no fluorescence was detected (Fig. 2e). Red fluorescence was observed for all of the CP fragment combinations except for those containing at least one F3-mRFP fusion (Fig. 2f). Immunodetection of transiently co-expressed mRFPN and mRFPC fusion proteins in plant leaves was performed with CP_{full}, F1 and F3 fusions (Zilian & Maiss, 2011) and the F2, F1_2 and F2_3 fusions (data not shown), and revealed that all of the fusion proteins were expressed in the inoculated leaves. In subsequent studies, the F3-mRFP and CP_{full}-mRFP fragments served as a non-interacting and an interacting control, respectively.

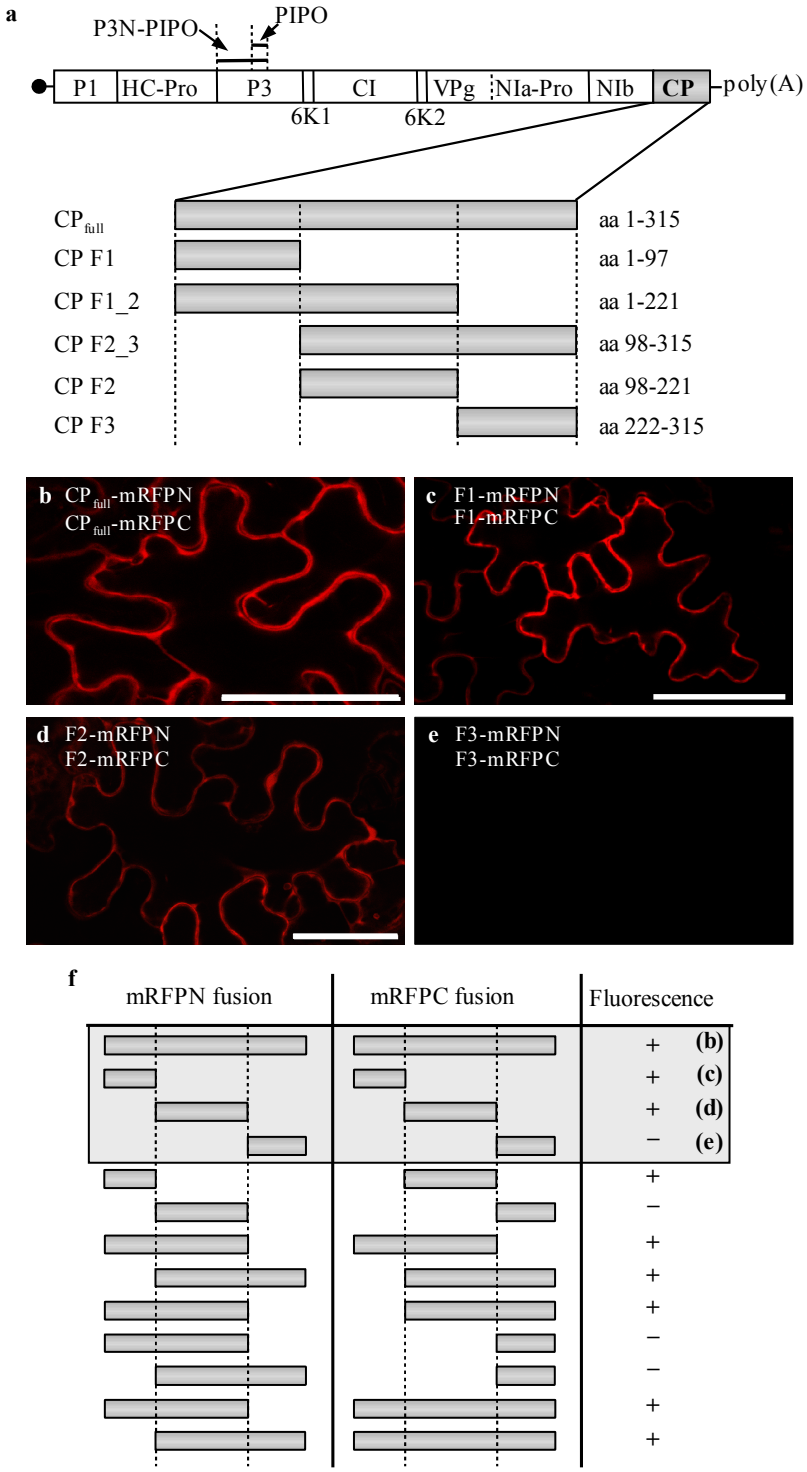

Fig. 2: Interactions of the full-length PPV_CP and PPV_CP deletion mutants. (a) Schematic depiction of the PPV genome organization. The ORF is represented by a box and the VPg at the 5'-end as a black circle, and the genes are indicated. P3N-PIPO and PIPO are marked by black bars. Below the genome organization is a map of the PPV_CP deletion mutants. Full-length CP (CP_{full}) was divided into three fragments by PCR mutagenesis, and five mutants were generated. CP_{full} and the F1, F1_2, F2_3, F2 and F3 fragments contain aa 1-315, 1-97, 1-221, 98-315, 98-221 and 222-315, respectively. (b-e) Interactions among CP_{full} and its mutants were tested by BiFC. CLSM images of the epidermal leaf cells co-inoculated with mixtures of agrobacteria harboring the expression plasmids pCB:CP_{full}-mRFPN and -mRFPC (b), pCB:F1-mRFPN and -mRFPC (c), pCB:F2-mRFPN and -mRFPC (d) and pCB:F3-mRFPN and -mRFPC (e); scale bars: 50 µm. (f) Schematic overview of the tested CP mutant combinations: + = fluorescence detected, - = fluorescence not detected.

PPV_CP interaction studies suggested that the F1 and F2 CP fragments contain interacting protein domains and that the C-terminal aa 222-315 were not involved in the self-interaction of PPV_CP. This is in contrast to the results of Kang *et al.* (2006), who reported the C-terminal aa 171-285 of SMV-G7H_CP to be important and sufficient for the self-interaction of the CP in a YTH approach. Studies on the assembly of TEV, *Johnsongrass mosaic virus* (JGMV) and PPV particles in *Escherichia coli* and *in planta* (Dolja *et al.*, 1991; Jagadish *et al.*, 1993, Dolja *et al.*, 1994; Jacquet *et al.*, 1998; Varrelmann & Maiss, 2000; Voloudakis *et al.*, 2003) have revealed two highly conserved aa motifs in the core region (RQ in FRQI) and the C-terminal part (D in FDFY) of the CPs, which are important for the assembly of particles. However, neither for SMV-G7H (Kang *et al.*, 2006) in a YTH nor for PPV in this BiFC assay, both, the core region and the C-terminal domain, were identified to be necessary for the self-interaction of the CPs. This may indicate that the aforementioned aa motifs are not essential for the physical interaction of single CP subunits, but are involved in building particle-like structures. Furthermore, the domains that are important for self-interaction do not seem to be conserved among potyviruses. Moreover, it is likely that the results of a BiFC assay more closely resemble the real conditions *in planta* than YTH approaches. To determine which aa motifs are necessary or sufficient for CP self-interactions, further detailed investigations with potyviral CPs are needed.

3.4.2 Interaction matrix of PPV proteins

An interaction matrix of ten proteins from PPV was generated using plasmids pBIN19:GOI-mRFPN and pBIN19:GOI-mRFPC to create N-terminal mRFP fusion proteins. Distinct full-length PPV genes were PCR-amplified from a PPV full-length clone (Maiss *et al.*, 1992) and integrated into the plant expression plasmids. To validate the expression of the mRFP fusion proteins, total protein extracts of inoculated *N. benthamiana* leaves were prepared at 3 dpi, and immunoblot analyses were performed using anti-c-myc and anti-HA antibodies to detect the mRFPN and mRFPC fusion proteins, respectively. All of the PPV proteins, including P3N-PIPO, were expressed at detectable levels (Fig. 3) and the estimated molecular weights were in accordance with the calculated ones. Additional protein bands of approximately 20 and 50 kDa were observed in all of the samples on the immunoblot probed with anti-c-myc antibodies.

These bands may have resulted from the non-specific binding of anti-c-myc antibodies to plant proteins. Additional bands of different sizes were detected with both antibodies for P1 and with the anti-c-cmyc antibodies for some other proteins, and may represent prematurely terminated proteins or proteolytic degradation products occurring during protein extraction. In case of P1 and NIa-Pro, these bands presumably represent fragments from proteinase activity.

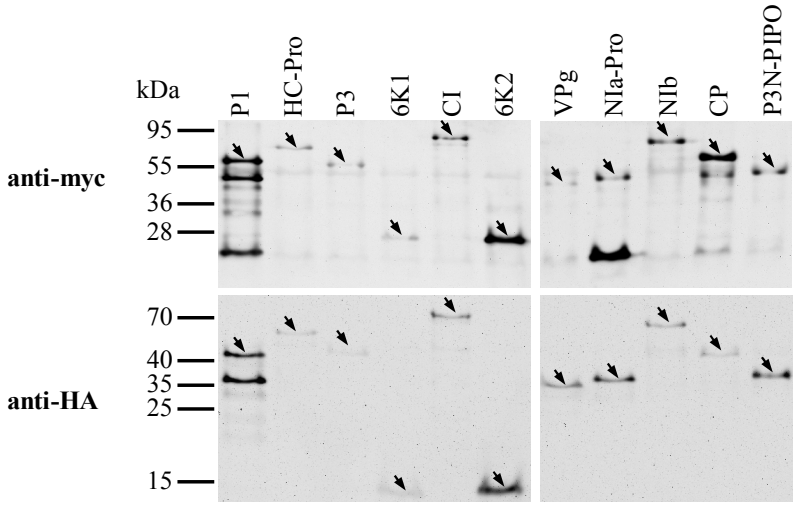

Fig. 3. Immunodetection confirming the expression of the mRFPN and mRFPC fusions with the PPV proteins in *N. benthamiana* leaves. The mRFPN fusion proteins were identified with anti-c-myc monoclonal antibodies and the mRFPC fusion proteins with anti-HA monoclonal antibodies in the immunoblot analyses of *N. benthamiana* leaf material 3 dpi. The analyzed fusion proteins are listed. A molecular mass ladder is indicated at the left. The molecular weight of the detected proteins was determined using the Spectra™ multicolor broad range protein ladder or the Page Ruler prestained protein ladder plus (Thermo Fisher). Arrowheads show the full-length fusion proteins at their calculated sizes.

The fluorescence of mRFP was visualized at 3 dpi in single leaf discs of inoculated leaves by CLSM. Each pair of viral proteins was tested at least twice and, additionally, the single proteins were fused to either the mRFPN or the mRFPC fragment, resulting in a total of 100 possible interaction combinations. The results are summarized in Tab. 2. Red fluorescence, representing interaction between the tested proteins, was detected for 52 protein combinations; among these the self-interaction of CI, 6K2, VPg, NIa-Pro, NIb and CP was observed. In addition, CI interacted with all of the other PPV proteins. VPg, NIa-Pro and NIb also interacted with at least six other proteins, whereas only a few interactions were observed for P1, HC-Pro and P3. The 6K proteins, not tested in all YTH systems (Guo *et al.*, 2001), were included in the experiments, and a 6K2 self-interaction and a 6K1/6K2 interaction were demonstrated for the first time.

In contrast to all previously reported interaction maps (Guo *et al.*, 2001; Kang *et al.*, 2004; Lin *et al.*, 2009; Shen *et al.*, 2010), more interactions were observed for PPV than for any other potyvirus. Lin *et al.* (2009) detected 39 interactions for SMV-P and 44 interactions for SYSV-O among the ten proteins, which differs from the results reported for PVA, PSbMV, SMV-G7H and PRSV-P (Guo *et al.*, 2001; Kang *et al.,* 2004; Shen *et al.,* 2010), where the number of identified interactions ranged from nine to 16. However, the results of the cited experiments may not represent the real situation in a plant. With regard to the wealth of functions of potyviral proteins (Urcuqui-Inchima *et al.*, 2001), a more complex interaction matrix would be expected, as is described in the present study and was reported by Lin *et al.* (2009).

Tab. 2: Protein interaction matrix for *Plum pox virus* (PPV) proteins generated with an optimized mRFP-based BiFC assay.

The mRFPN fusion proteins are indicated at the left side, the mRFPC fusion proteins on top of the table: + = fluorescence detected (additionally highlighted in yellow), - = fluorescence not detected. Results of self-interaction tests are boxed.

		mRFPC fusions									
		P1	**HC-Pro**	**P3**	**6K1**	**CI**	**6K2**	**VPg**	**NIa-Pro**	**NIb**	**CP**
mRFPN fusions	**P1**	-	-	-	-	+	-	+	+	-	+
	HC-Pro	-	-	-	-	+	-	-	-	-	-
	P3	-	-	-	-	+	-	-	+	+	-
	6K1	-	-	-	-	+	+	-	+	-	-
	CI	-	+	+	+	+	+	+	+	+	+
	6K2	-	-	-	+	+	+	+	+	+	-
	VPg	-	-	-	-	+	+	+	+	+	+
	NIa-Pro	-	-	+	+	+	+	+	+	+	+
	NIb	-	-	+	-	+	+	+	+	+	+
	CP	-	-	-	-	+	-	+	+	+	+

Particularly remarkable is the number of interactions detected for CI. López *et al.* (2001) tested the PPV_CI self-interaction and interaction with NIa-Pro, NIb, CP and P3/6K1 in a YTH approach, but the authors failed to detect any interaction. The present BiFC study clearly demonstrates a CI self-interaction as well as interactions with all other PPV proteins directly *in planta.* In the YTH approach (López *et al.*, 2001), only single CI deletion mutants revealed the self-interaction of different CI domains, and the

authors suggested that the full-length CI possibly requires additional virus or plant factors to interact. Indeed, a missing interaction may have possibly resulted from the limitations of the YTH system, as the CI protein may require *in planta* conditions for an interaction. However, this negative result is in contrast to the results obtained with SMV-P and SYSV-O CIs (Lin *et al.,* 2009) in YTH assays, which demonstrated the interactions of the CIs with themselves and other proteins. Although it is possible that some homologous proteins of two viruses might differ in their interaction behavior, the results of the two different studies concerning PPV_CI interactions suggest that some interactions of certain proteins occurring *in planta* may not be detectable in yeast cells. Specifically, the detection of interactions between large proteins has often failed in YTH approaches due to protein instability or blocked interaction domains (López *et al.,* 2001). Furthermore, protein interactions in yeast cells depend on localization of the proteins in the nucleus, which represents a significant limitation of the method (Golemis *et al.*, 1999) with regard to *in planta* systems. Since proteins, which are localized in other cell compartments than the nucleus and may contain a nuclear export signal, are not able to activate transcription of the reporter gene in the nucleus, the number of false negative results is increased.

Expect for P1, all of the interactions reported here were observed regardless of whether the proteins were fused to the mRFPN or the mRFPC fragment. In fact, interaction of P1 individually with CI, VPg, NIa-Pro and CP was observed only when P1 was fused to the mRFPN and the other proteins fused to the mRFPC fragment. No interaction with these proteins as mRFPN fusions was detected when P1 was fused to the mRFPC fragment. As the interaction of P1 and CI has been reported previously for *Potato virus Y* (PVY) (Arbatova *et al.*, 1998), PVA (Guo *et al.*, 1999; Merits *et al.*, 1999) and SYSV-O (Lin *et al.*, 2009), it is evident that P1 interacts with CI and also with VPg, NIa-Pro and CP. Certainly, P1 and P3 are less conserved among potyviral proteins (Shukla *et al.*, 1991), and PPV_P1 may have interaction partners other than the P1 proteins of other potyviruses. However, single protein interactions can depend on the type of fusion created, as has been observed previously for other potyviruses in nearly every YTH approach. The yeast-based systems seem much more predisposed to such directionality than the BiFC assay and often produce inconsistent results. Guo *et al.* (2001) suggested that some protein fusions may have more favorable protein folding, which may also be true in BiFC approaches. It has also been reported for some BiFC studies that single

proteins show such directionality (Bracha-Drori *et al.*, 2004; Citovsky *et al.*, 2008), and it cannot be excluded that, in single cases, as described here for P1, deficient protein folding can possibly interfere in the interactions of proteins of interest.

Kerppola (2006) described the necessity to determine the optimal arrangement of the fusion proteins to enable the fragments of the reporter to associate with each other. To exclude that missing interactions in this study resulted from an insufficient BiFC system, single PPV protein combinations, including P3-P3, P3-6K2 and CP-HC-Pro, were additionally tested for interaction with pCB:mRFPN-GOI and pCB:mRFPC-GOI, which give C-terminal mRFP fusion proteins. All of the tested combinations revealed results that were identical to those obtained with the plasmids encoding the N-terminal fusions (data not shown), thus indicating that the order of the fusion proteins had no impact on the possible interactions. However, in case of C-terminal mRFP fusions the detected fluorescence signals were often weaker (data not shown).

In addition to the detection of interactions between certain proteins, the BiFC system offers the opportunity to localize protein interactions. The diversity of the protein interactions is illustrated in Fig. 4, showing the BiFC fluorescence of the proteins (CI, 6K2, VPg, NIa and NIb) that self-interacted, and the self-interaction of PPV_CP is demonstrated in Fig. 2b. Red fluorescence, representing a CI self-interaction (Fig. 4a), was restricted to small aggregates along the cell walls of epidermal cells, presumably representing plasmodesmata (Wei *et al.,* 2010b). For 6K2 self-interaction fluorescence in vesicular structures resembling chloroplasts was observed (Wei *et al.*, 2010a; Fig. 4b). The red fluorescence of the VPg self-interaction was observed predominantly in the nucleus of cells (Fig. 4c), with an accumulation in the nucleolus, reflecting the nuclear localization of VPg, as has been demonstrated for different potyviruses (Beauchemin *et al.,* 2007, Schaad *et al.,* 1996). An accumulation of fluorescence in nuclei and in the cytoplasm of epidermal cells was observed after the co-expression of NIa-Pro-mRFP fusions (Fig. 4d), and fluorescence deriving from NIb self-interaction was detected in small aggregates along the cell walls (Fig. 4e).

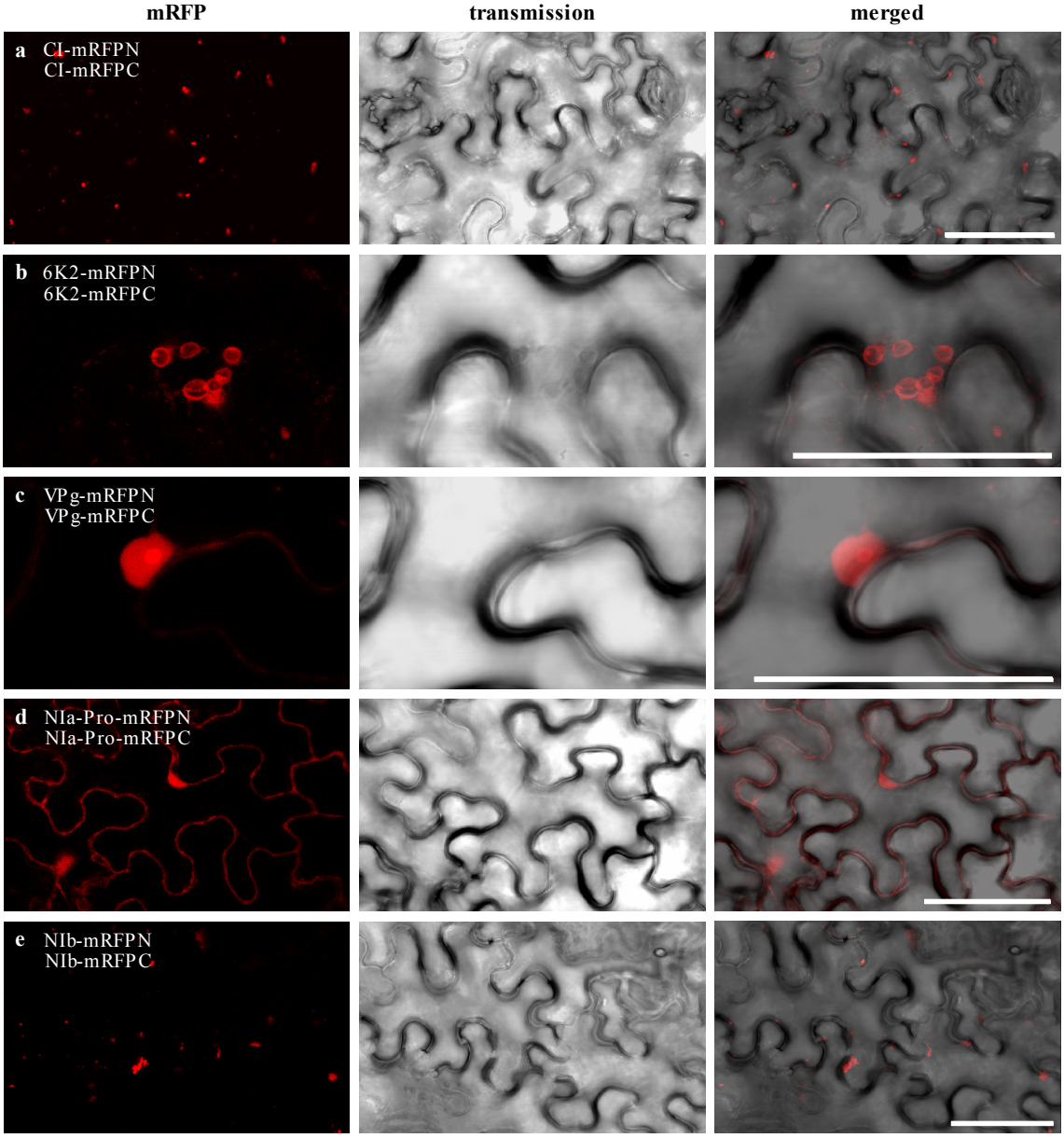

Fig. 4: Self-interactions of the PPV proteins. BiFC of mRFP in *N. benthamiana* epidermal cells at 3 dpi. CLSM images for the mRFP fluorescence, the transmitted light mode and merged pictures of cells co-inoculated with pBIN19:CI-mRFPN and -mRFPC (a), pBIN19:6K2-mRFPN and -mRFPC (b), pBIN19:VPg-mRFPN and -mRFPC (c), pBIN19:NIa-Pro-mRFPN and -mRFPC (d) and pBIN19:NIb-mRFPN and -mRFPC (e). Scale bars: 50 µm.

Chung *et al.* (2008) described an additional ORF (PIPO) embedded in the P3 cistron of TuMV and other potyviruses, encoding a P3N-PIPO fusion protein. Previously, an interaction of P3N-PIPO and CI was identified by BiFC assay for TuMV and was proposed to be essential for cell-to-cell movement (Wei *et al.*, 2010b). Within this study, the localization of these proteins in plasmodesmata was determined using fluorescent reference markers. Here, P3N-PIPO-CI and P3N-PIPO-CP interactions were investigated by using BiFC. The expression of the P3N-PIPO-mRFP fusions was

verified by immunoblot analyses (Fig. 3), and BiFC was performed using both plasmid sets, encoding either the N- or C-terminal mRFP fusion proteins.

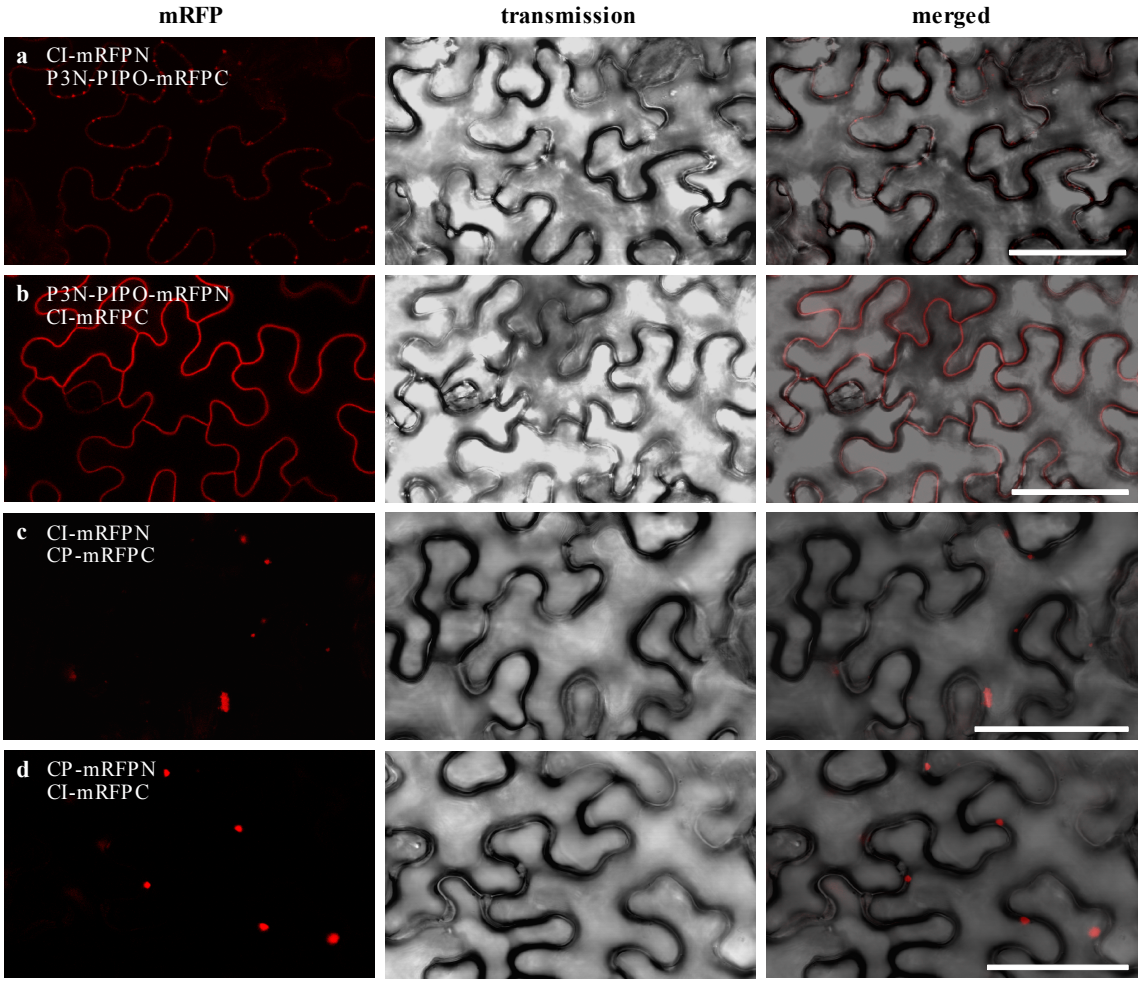

Fig. 5: Interactions of PPV_CI, PPV_P3N-PIPO and PPV_CP. CLSM images for the mRFP fluorescence, the transmitted light mode and merged pictures of cells co-inoculated with pCB:P3N-PIPO-mRFPN and pCB:CI-mRFPC (a), pCB:CI-mRFPN and pCB:P3N-PIPO-mRFPC (b), pCB:CI-mRFPN and pCB:CP-mRFPC (c) and pCB:CP-mRFPN and CI-mRFPC (d). Scale bars: 50 µm.

The co-expression of P3N-PIPO and CI resulted in fluorescence complementation (Fig. 5a and b), predominantly within distinct puncta along the cell walls of epidermal cells (Fig. 5a), which could indicate plasmodesmal localization, as has been described for TuMV by Wei *et al.* (2010a). However, the finding requires confirmation by the use of appropriate localization markers in additional studies. The CP and CI interaction was analyzed further (Fig. 5c and d), revealing single fluorescent aggregates in epidermal cells that were localized along the cell walls. However, neither the self-interaction of P3N-PIPO nor the interaction of P3N-PIPO with CP was observed (data not shown). These results extend the findings of Wei *et al.* (2010b), who neither analyzed a possible

self-interaction of P3N-PIPO nor investigated an interaction of P3N-PIPO and CP, which is also involved in cell-to-cell movement. All of the results obtained for PPV_P3N-PIPO in this study support the recently postulated model for potyvirus intracellular transport through plasmodesmata, whereby P3N-PIPO interacts physically with CI, and CI interacts with CP.

The results and those of others illustrate that, in addition to the detection of physical protein-protein interactions, BiFC has the capability to resolve the localization of proteins at the subcellular level (Bracha-Drori *et al.,* 2004; Citovsky *et al.*, 2006, 2008; Martin *et al.*, 2009), a feature that a YTH assay cannot provide. However, the confirmation of subcellular localization requires supplementation with fluorescent reference marker, which are co-expressed with the fusion proteins (Citovsky *et al.*, 2008).

New and known potyviral protein interactions were demonstrated with the BiFC system. Surprisingly, neither an interaction of HC-Pro and CP nor a self-interaction of HC-Pro could be verified; however, because an interaction between CP and HC-Pro is essential for aphid-transmission (Atreya & Pirone, 1993; Blanc *et al.*, 1997, 1998; Peng *et al.*, 1998), an interaction of these two proteins is very likely. However, this interaction has not been consistently reported for all potyviruses (Guo *et al.*, 2001; Shen *et al.*, 2010) and, therefore, the interaction of these two PPV proteins *in planta* is not obvious. Nevertheless, self-interaction of HC-Pro was expected, as has been described previously for different potyviruses using YTH assays (Guo *et al.*, 1999, Urcuqui-Inchima *et al.*, 1999), but it could not be demonstrated for full-length PPV_HC-Pro in this *in planta* BiFC. Therefore, more detailed experiments with PPV_HC-Pro were conducted.

3.4.3 Full-length PPV_HC-Pro self-interaction was not detected *in planta,* whereas the N- and C-terminal domains interact with themselves

Zheng *et al.* (2010) analyzed TuMV_HC-Pro with regard to self-interaction and mapped different domains that were involved in this interaction by YFP-based BiFC *in planta*. This report was the first BiFC assay to demonstrate that a potyviral HC-Pro self-interacts *in planta*. To confirm that the lack of PPV_HC-Pro self-interaction did not depend on the BiFC system, deletion analyses with PPV_HC-Pro and TuMV_HC-Pro were performed. TuMV_HC-Pro was RT-PCR-amplified from the total RNA extracted

from a TuMV [DSMZ PV-0104]-infected *N. benthamiana* plant and integrated into BiFC plasmids, giving rise to pCB:TuMV_HC-Pro-mRFPN and -mRFPC. According to the report of Zheng *et al.* (2010), PPV_HC-Pro and TuMV_HC-Pro were divided into two domains, thus generating the deletion mutants TuMV_HC-ProN and PPV_HC-ProN, which consist of aa 1-99, and TuMV_HC-ProC and PPV_HC-ProC, which consist of aa 100-458 of the HC-Pros (Fig. 6a). To analyze the self-interaction of the full-length proteins and their mutants, *N. benthamiana* plants were inoculated, and the fluorescence was recorded at 3 dpi by CLSM (Fig. 6b-f).

Fluorescence was detected in the *N. benthamiana* epidermal cells that were co-expressing TuMV_HC-Pro-mRFP fusions in small aggregates along the cell walls (Fig. 6b), which confirmed the self-interaction of the TuMV_HC-Pro *in planta.* Moreover, the co-inoculation of the TuMV_HC-ProN-mRFP fragments and the TuMV_HC-ProC-mRFP fragments revealed fluorescence (Fig. 6c and d), whereas the self-interaction of PPV_HC-Pro was not demonstrated (data not shown). However, the fluorescence was detected as irregular aggregates in plant cells co-expressing the PPV_HC-ProN-mRFP and PPV_HC-ProC-mRFP fusions (Fig. 6e and f), demonstrating clearly that the lack of self-interaction of the full-length PPV_HC-Pro did not result from an insufficient BiFC system.

Many studies have focused on HC-Pro of different potyviruses, also demonstrating self-interaction (Urcuqui-Inchima *et al.*, 1999; Guo *et al.*, 2001; Plisson *et al.*, 2003; Yambao *et al.*, 2003; Kang *et al.*, 2004; Lin *et al.*, 2009; Shen *et al.*, 2010). Moreover, it has been proposed that HC-Pro acts as a homo-dimer in different stages of the virus infection cycle (Thornbury *et al.,*1985; Urcuqui-Inchima *et al.,*1999; Wang & Pirone*,* 1999) and that at least two HC-Pro subunits are necessary for an interaction with CP oligomers of (Ruiz-Ferrer *et al.* 2005). All of these studies have led to the conclusions that HC-Pro acts as dimer and that physical interaction of single HC-Pro subunits occurs. However, most of these studies were performed with YTH systems. Here, the PPV_HC-Pro self-interaction was not demonstrated *in planta*, and it is not certain whether PPV_HC-Pro exists as a dimer during virus replication. Self-interaction has not been demonstrated for PPV_HC-Pro *in planta.*

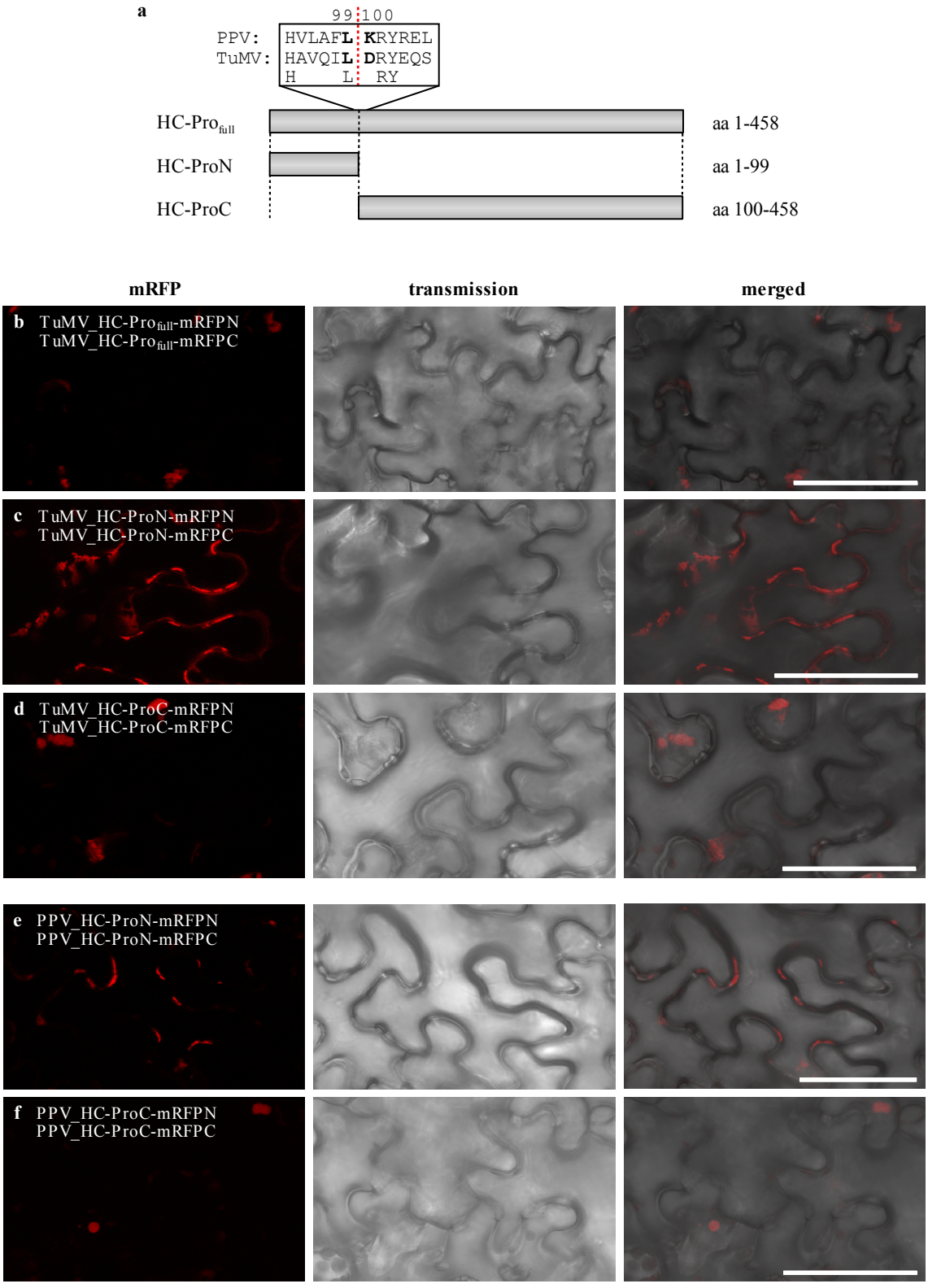

Fig. 6. Interactions of full-length TuMV_HC-Pro, PPV_HC-Pro and their mutants. (a) Map of the TuMV_HC-Pro and PPV_HC-pro deletion mutants. Full-length HC-Pros were divided into two fragments by PCR mutagenesis. The HC-Pro fragments N and C contain aa 1-99 and aa 100-458, respectively. (b-f) CLSM images for the mRFP fluorescence, the transmitted light mode and merged pictures of cells co-inoculated with TuMV_HC-Pro-mRFPN and -mRFPC (b), TuMV_HC-ProN-mRFPN and -mRFPC (c) and TuMV_HC-ProC-mRFPN and -mRFPC (d), PPV_HC-ProN-mRFPN and -mRFPC (e) and PPV_HC-ProC-mRFPN and -mRFPC (f). Scale bars: 50 µm.

Furthermore, experiments with the HC-Pros of two additional PPV strains (an aphid-transmissible strain, PPV-AT, and a Bulgarian strain of PPV [DSMZ, PV-0212]) and *Potato virus Y* (PVY-N605; Jakab *et al.*, 1997) revealed no self-interaction of the respective HC-Pros (data not shown), whereas the TuMV_HC-Pro (Zheng *et al.*, 2010) self-interaction was verified by the mRFP-based BiFC system. It is possible that not all full-length HC-Pros self-interact *in planta*. These data, together with the lack of interaction between the HC-Pros of PPV-NAT, PPV-AT, PPV-BUL and PVY-N605, support a species-specific or strain-specific HC-Pro interaction.

In conclusion, BiFC is a very useful and reliable system for the detection and localization of potyviral protein interactions *in planta* and complements existing YTH maps. Although YTH systems are powerful tools for the study of potyviral protein interactions, BiFC assays *in planta* represent more natural conditions than studies in yeast cells. Our findings for PPV proteins revealed complex and diverse interactions that are probably not conserved among all of the species in the genus *Potyvirus*. However, further research, including analyses of protein structures, more detailed mutational analyses and BiFC studies in virus-infected plant cells, will help to clarify the participation of PPV proteins in the infection cycle.

4 General discussion

This study was launched to generate a complete protein interaction matrix of the PPV proteins *in planta*. For this purpose, an existing BiFC system was optimized. As the YTH system represents the dominant tool for revealing the complex networks of proteins of numerous organisms and viruses it has been the method of choice for many research groups to get insight into the interaction networks of potyviral proteins (Hong *et al.*, 1995; Li *et al.*, 1997; Guo *et al.*, 1999; Merits *et al.*, 1999; Urcuqui-Inchima *et al.*, 1999; Choi *et al.*, 2000; López *et al.*, 2001; Roudet-Tavert *et al.*, 2002; Yambao *et al.*, 2003; Seo *et al.*, 2010). Nevertheless, this yeast technology as well as other methods has certain limitations, and the improvement of the systems and a persistent development of further methods was pursued providing new technologies with additional features. These include fluorophore-based systems like BiFC, which enable a visualization of interactions directly in living cells. Employing such BiFC system, a complete interaction map for PPV proteins was generated in *N. benthamiana* as the experimental host plant, which represents the first BiFC-based interaction map ever published for a potyvirus. Comparisons with maps of other potyviruses were made and this all together should help in understanding the complex interplay between potyviral proteins during the infection cycle.

The first part of this work focused on the optimization of a recently developed mRFP-based BiFC system (Jach *et al.*, 2006) for the detection of the viral protein-protein interactions in living plant cells. Since the introduction of a YFP-based BiFC as a new approach in proteomic research by Hu *et al.* (2002) more than ten fluorescent proteins have become available for BiFC (Shyu & Hu, 2008). The number of modified or improved systems is continuously increasing (Kodama, 2011), because manipulation of the method is easy and thus allow an optimal adjustment to a certain investigation. In 2006 Jach *et al.* reported the development of a red BiFC system based on an improved mRFP1, and thus added red to the spectral possibilities of BiFC. Whereas DsRed widely used as reporter protein is known to tetramerize (Bevis & Glick, 2002) and therefore impede its application in BiFC, mRFP1 (Campbell *et al.*, 2002), as a monomeric variant, is suitable for use in BiFC. Jach *et al.* (2006) generated a mutated form of mRFP1 (mRFP1-Q66T), which spectral properties resembling more the DsRed than the wildtype mRFP1. In comparison to mRFP1, mRFP1-Q66T was shown to be an mRFP

with fast maturation time, high photostability and high brightness comparable to that of the reporter protein DsRed (Jach *et al.*, 2006). Moreover, the use of mRFP1-Q66T is of practical advantage. Since the excitation and emission peak of this variant is shifted towards those of DsRed, standard microscopic fluorescence filters for DsRed can be used in fluorescence microscopy and CLSM. Jach *et al.* (2006) presented a very promising system using the mRFP1-Q66T, which was the method of choice to analyze viral protein-protein interactions in this work.

Here, new mRFP1-Q66T BiFC plasmids were developed employing single components of the original system as well as new ones. This plasmid optimization included three main steps, which were (1) the removal of the Gateway®-cloning sites in favor of classical restriction endonuclease cleavage sites, (2) the introduction of a special glycine/serine-linker (GGGSGGG) and (3) the introduction of a smaller vector-backbone. Moreover, two different plasmid sets were generated enabling the investigation of N- and C-terminal mRFP fusions representing an additional feature and improvement over the original system. In all experiments that were performed for every single optimization step, the functionality of the modified system was clearly demonstrated.

Nevertheless, although modification of a system seems to be easily feasible certain aspects have to be kept in mind for optimizing and performing BiFC analyses. Single changes of the system, as it is generally true for all methods, always implicate that controls, negative as well as positive controls, have to be adopted (Kerppola, 2006). Appropriate non-interacting and interacting controls for BiFC are a basic requirement for a correct determination of specific protein interactions (Ohad *et al.*, 2007; Citovsky *et al.*, 2008). Appropriate proteins for positive controls widely used in *in planta* BiFC are bZIP- (basic leucine zipper) transcriptional activators (Hu *et al.*, 2002; Stolpe *et al*., 2005; Walter *et al.*, 2004), dimerizing fluorescent proteins with other spectral properties than the chosen BiFC fluorophore (Jach *et al*., 2006; Fan *et al*., 2008) or numerous other plant proteins known to di- or multimerize (for an overview see Bhat *et al.*, 2006; Kerppola *et al.*, 2006). As the aim of this study was the investigation of plant viral proteins, a plant viral protein should be engaged to function as positive control. PPV_CP as structural protein is involved in particle assembly, which implies an interaction of CPs. Moreover, self-interaction of some potyviral CPs has already been demonstrated in different studies (Guo *et al.*, 2001; Kang *et al.*, 2004,

2006; Lin *et al.*, 2009; Shen *et al.*, 2010). In consequence, PPV_CP seemed to be the optimal interacting control for this study. The obtained results for CP-mRFPN and CP-mRFPC co-expression in plant cells clearly approved the capability of PPV_CP to function as positive control in this system. While entire proteins can be used as positive controls in BiFC, optimal non-interacting controls were described as proteins in which the interaction domain has been mutated or deleted (Kerppola, 2006). Since PPV_CP revealed to be a reliable positive control, a CP mutant should be utilized as a non-interacting control. Based on the results of Kang *et al.* (2006) for SMV_CP, different PPV_CP mutants were generated. In experiments with these CP mutants a single domain comprising of aa 222-315 of the protein, CP_F3, was identified to be not involved in the self-interaction of the protein and was used in this study, and can be used in future analyses, as non-interacting control.

Once appropriate proteins were identified as controls, further points had to be considered upon the optimization of the BiFC system. Fluorescent protein fragments are able to reconstitute functional fluorescent complexes with a low efficiency in the absence of specific interaction (Kerppola, 2006) depending on the fluorescent protein and the point of time after infiltration of the plants. This is very critical for the evaluation of results and two main points had to be considered.

Firstly, a time frame for CLSM, that means a time in which fluorescence signals can be clearly identified as result of a real interaction, had to be determined. At different timepoints after inoculation with the interacting and the non-interacting control, respectively, discs of inoculated leaves were assayed for fluorescence to evaluate the time at which fluorescence can be detected in the positive as well as in the negative control. The optimal time frame for the investigation with this BiFC system revealed to be 68-78 h past inoculation, that means at day 3 past inoculation. Most protein combinations revealed at least weak fluorescence 4-6 dpi implicating false positive results (data not shown). Consequently, all approaches here were performed 3 dpi. Such limited duration of only few hours or days for the performance of BiFC was already reported in other studies (Kerppola, 2006; Kim *et al*., 2007; Ohad *et al.*, 2007). The determination of the time interval between complete maturation of the fluorescent protein and the occurrence of false positive interactions was described to be critical. Moreover, this interval for detection of real interactions is the reason why BiFC does not enable the observation of the interaction dynamics (Kerppola, 2008). On the one

hand, maturation of the fluorophore has to be completed before a detection of protein interaction is possible. On the other hand, a detection of interactions is restricted to the point of time from which unspecific complex formation occurs. Moreover, the reconstitution of the bimolecular fluorescent complex is at least *in vitro* essentially irreversible (Hu *et al*., 2002; Magliery *et al*., 2005; Kerppola, 2006), which additionally impede the observation of protein interaction dynamics.

Secondly, mRFP fusion proteins had to be verified for an autoactivation. Autoactivation can be described as the reconstitution of the fluorophore complex without a specific interaction of two proteins. To validate this, here a 17-aa peptide, referred to as an MCS, fused to the one mRFP fragment and PPV_CP fused to the other mRFP fragment were co-expressed *in planta*. No fluorescence was detected 3 dpi, which demonstrated that mRFP fragments could not reconstitute a functional fluorophore upon missing protein interaction. The use of a strong promoter for expression of the fusion proteins might be critical and possibly promotes an reconstitution of the fluorophore complex without interaction of proteins (Walter e*t al.*, 2004; Caplan *et al*., 2008; Citovsky *et al*., 2008; Kerppola, 2008). As demonstrated in the control experiments the use of the strong 35S promoter in this system had certainly no negative effect on the interaction studies.

Finally, to complete the optimization of the system considering all mentioned aspects additional proteins of plant viruses from different genera were used. All experimental results obtained with CaCV_N, TMV_CP, TYLCTHV_BV1 and TYLCTHV_BC1 corresponded with the expectations. Conclusively, this reaffirmed that once established, the BiFC in general and this optimized mRFP-based BiFC in special, per se represents a reliable and manageable method for the detection of single protein-protein interactions *in planta* and a powerful alternative to other methods like YTH systems.

In the second part of this work, the optimized BiFC system was employed to investigate the protein interactions of the eleven PPV proteins, thus the implementation of the system to reveal distinct protein interactions was in the focus of research. In a first step the development of the interacting and non-interacting control from the first part of the work was taken up again. Since the results for F1 and F3 mutants of the PPV_CP did not correspond with results obtained in an investigation of interactions between different SMV_CP mutants (Kang *et al.*, 2006), a closer look at PPV_CP was taken. Results clearly revealed that the C-terminal fragment F3 (aa 222-315) of PPV_CP is not necessary for a self-interaction, but that the N-terminal fragment F1 (aa 1-97) or the

core region F2 (aa 98-221) alone are sufficient to affect self-interaction. For SMV exactly the F3 fragment was identified in a YTH system to be necessary for the self-interaction of the CP (Kang *et al.*, 2006). So the question which was raised here is, whether these controversial findings were a result of methodological discrepancy or whether potyviral CPs really differentiate in their domain for self-interaction. Baratova *et al.* (2001) performed some experiments on the CP of PVA to determine the tertiary structure of the protein. The authors suggested that aa 129-137, localized in the central part of the protein, may play an important role in oligomerization of PVA_CP, supporting the results obtained for PPV_CP in this study. Suggesting that the results for SMV_CP reflect the natural occurrence *in planta*, this all together indicate that the domain for CP self-interaction is not conserved among potyviruses.

Actually, different interaction domains of homologous potyviral proteins have already been reported for the HC-Pro. HC-Pro of different potyviruses has often been in focus of research and was suggested to interact with itself (Urcuqui-Inchima *et al.*, 1999; Guo *et al.*, 2001; Plisson *et al.*, 2003; Yambao *et al.*, 2003; Kang *et al.*, 2004; Lin *et al.*, 2009; Shen *et al.*, 2010). While the domain for HC-Pro self-interaction was mapped for PVY to the N-terminal part of the protein (Urcuqui-Inchima *et al.*, 1999) and for PVA to an N- as well as a C-terminal domain (Guo *et al.*, 1999) in YTH approaches, Zheng *et al.* (2010) reported that the central and C-terminal regions of TuMV_HC-Pro participate in self-interaction in a YTH approach and additionally the N-terminal region in a BiFC. Although, HC-Pro was investigated to a broader extent, and the results will be discussed at a later time, the observations on different potyviral HC-Pros corroborate the hypothesis of possible different interaction domains for homologous potyviral proteins. Nevertheless, it cannot be excluded, that, as already suggested by Zheng *et al.* (2010), in general also methodological limitations cause such inconsistent results. The detection of different interaction domains for PPV_CP and SMV_CP may reflect this problem. Results obtained from BiFC assay *in planta* probably reflect a more natural situation than those from YTH approaches and therefore, the results from these two methods do not necessarily correspond with each other.

The major objective of this work was the generation of an interaction map of the PPV proteins P1, HC-Pro, P3, 6K1, CI, 6K2, VPg, NIa-Pro, NIb and CP. Employing the previously developed mRFP-based BiFC system such matrix was compiled. For 52 out of 100 tested protein combinations, fluorescence was observed, indicating protein-

protein interactions. Fluorescence was clearly detectable and the results were reproducible. 52 interactions are more interactions than ever detected for any potyvirus, and this work provides the most exhaustive view of the PPV encoded proteins´ interactome ever published. The results of the PPV interaction study by BiFC raise two hypothesis on the conspicuous differences between interaction maps of different potyviruses. 1. BiFC seems to be a more reliable system than the YTH systems. Employing the BiFC *in planta* more interactions were detected under more natural conditions than in yeast cells. In addition, YTH assays have limitations, like the requirement for a nuclear localization of proteins, which may cause false negative interactions. 2. Homologous proteins of different viruses of the genus *Potyvirus* reveal a different interaction behavior, so that more PPV proteins interact with each other than proteins of other viruses.

However, to make an exact prediction additional research is needed. The parallel investigation of a number of different viruses under identical conditions with the same method may help to clarify these assumptions. Lin *et al.* (2009) conducted parallel YTH experiments with SMV-P and SYSV-O. However, the results did not allow the identification of identical patterns between protein interactions of the two viruses. 39 interactions were detected for SMV-P and 44 interactions for SYSV-O and the interaction patterns clearly differ. At the first site these results rather support the second assumption of a non conserved interaction behavior of potyviral proteins. However, due to a further limitation of the system a direct comparison of found interactions is difficult. In several cases, as is is was also true for all other interaction maps developed with YTH systems (Guo *et al.*, 2001; Kang *et al.*, 2004; Shen *et al.*, 2010), interactions between different proteins were only detected, when the one protein was fused to the AD and the second protein was expressed in fusion with the DBD, but not *vice versa.* This can be described as some kind of directionality of interactions. Therefore, on the one hand the idea to conduct parallel experiments with different potyviruses is a possibility to collect data on potyviral interaction behavior. On the other hand, the yeast system seems to be a limited technology for this, since it is not clear whether numerous interactions, which are found only in one direction, are true interactions or not. The BiFC system revealed also such directionality, but only for four reactions with the protein P1. Interactions between P1 and CI, VPg, NIa-Pro and CP, respectively, were detected only when P1 was expressed in fusion with the mRFPN and the other proteins

in fusion with the mRFPC fragment. Nonetheless, it would be interesting to see, whether parallel BiFC experiments with different potyviruses deliver more consistent results.

Moreover, a comparison between interactions of PPV_CI detected in a YTH approach (López *et al.*, 2001) and the results for PPV_CI observed in a BiFC in this study strengthen the first theory. As CI aggregates in the cytoplasm of infected cells forming typical inclusions, it is suggested that the protein is able to self-interact. However, López *et al.* (2001) could not detect a CI self-interaction with a YTH system. Furthermore, CI was analyzed in combination with the PPV proteins P3/6K1, CP, NIa-Pro and NIb, but with negative results for all the tested combinations. In contrast, in this work CI was demonstrated to interact with all PPV proteins, including CI itself and P3N-PIPO. Results of the CI interactions correspond with the expectations. As CI is involved in cell-to-cell movement (Carrington *et al.*, 1998), CI is suggested to interact with itself, CP and P3N-PIPO in the process of virion recruitment to plasmodesmata (Wei *et al.*, 2010b; Niehl & Heinlein, 2011), which will be discussed in a later part of this chapter. Furthermore, as an RNA helicase (Laín *et al.*, 1990), CI is part of the potyviral replication complex in genome replication (Fernández *et al.*, 1997) and this participation in genome replication suggests an interaction with other proteins of the complex. These include NIa-Pro, NIb, 6K2 and VPg. Guo *et al.* (2001) could not detect any of these interactions, neither for PVA nor for PSbMV, in YTH approaches and suggested that the recruitment of CI to the replication complex might be regulated by its RNA-binding features. The obtained results support a hypothesis of direct interactions between the proteins.

Quite remarkable is the appearance of a cluster of many interacting proteins in the C-terminal part of the PPV genome involving the proteins CI, 6K2, VPg, NIa-Pro, NIb and CP, which all interact with each other (with one exception: CP and 6K2 did not interact with each other). As already mentioned, these proteins, excluding CP, form the replication complex and participate in genome replication. A model for this process has been described. The 6K2 protein is suggested to anchor the replication complex at the ER-like membranes (Schaad *et al.*, 1997a), and an interaction with VPg, the putative primer for RNA synthesis (Murphy *et al.*, 1996), and NIa-Pro is very likely. The RdRP (NIb) may directly contact the VPg in close proximity to the RNA attachment site (Hong *et al.*, 1995) after its recruitment to the complex by NIa-Pro. Furthermore, as CP

was detected to interact with NIb it was suggested to play also a role in regulation of viral RNA synthesis (Mahajan *et al.*, 1996; Urcuqui-Inchima *et al.*, 2001). Conclusively, a complex interplay between the proteins by physical interactions seems to be very obvious. Single potyviral protein interaction maps reflect these results in a similar way (PSbMV, Guo *et al*., 2001; SMV-P, Lin *et al*., 2009), but for other viruses only single interactions could be detected (SMV-G7H; Kang *et al.*, 2004), which again demonstrate the inconsistence of YTH results.

For P1, as already mentioned above, interactions with CI, VPg and NIa-Pro were detected, however, only when P1 was expressed in fusion with the mRFPN fragment. As described in chapter 3 a methodological limitation cannot be excluded and deficient protein folding may be a reason for this phenomenon. However, it is remarkable, that such directionality was only monitored for P1. This is in contrast to YTH assays, where directionality of interactions is a great problem in analyzing results. SMV-P interaction studies with a YTH assay resulted in 39 positive interactions out of 100 possible (Lin *et al.*, 2009). However, 10 of these 39 interactions were detected only in one direction, that means, a certain protein interacted when it was fused to the AD but not as a fusion with the DBD. So the question is, whether these interactions are true interactions and the complementary pairs false negatives or *vice versa*. Structural and mutational analysis of proteins and protein complexes might help in making predictions about the occurrence of real interactions. As mentioned above, Baratova *et al.* (2001) conducted structural analysis on PVA_CP and developed a three-dimensional model of the protein. Due to this model, Baratova *et al.* (2001) made predictions about an oligomerization domain of the protein. However, these results have not yet approved in interaction studies with PVA_CP and mutants thereof. Nevertheless, apart from the fact that structural analysis are technically complex, they might help in understanding the interplay between proteins. Some BiFC studies also report such directionality of interactions (Bracha-Drori *et al.*, 2004; Citovsky *et al.*, 2008), and it therefore cannot be excluded that also in BiFC deficient protein folding may have influence on the interactions of proteins.

P1 may really interact with CI as it was reported for PVY (Arbatova *et al.*, 1998), PVA (Guo *et al.*, 1999; Merits *et al.*, 1999) and SYSV-O (Lin *et al.*, 2009). Since its description as accessory factor in genome amplification (Verchot *et al.*, 1995) an interaction with CI as well as VPg, NIa-Pro and NIb is possible. Furthermore, P1 is

suggested to be associated with symptomatology (Wisler *et al*., 1995) and important in defining the potyvirus´ host range (Salvador *et al*., 2008). Possibly an interaction with CP is needed, but the exact mechanism has not yet been revealed. The functions of P3, the second less conserved potyviral protein are also not well understood. Here, a P3 interaction with CI, NIa-Pro, NIb and CP was detected. Rodríguez-Cerezo *et al.* (1993), identified the TVMV_P3 in association with cylindrical inclusions again suggesting a participation of a further potyviral protein in genome replication (Merits *et al.*, 2002). As the P3 protein was identified as a pathogenicity determinant (Jenner *et al*., 2003; Suehiro *et al*., 2004) interactions with other proteins is very likely.

Complete novel findings in this study were the self-interaction of 6K2 and an interaction between 6K1 and 6K2. First published data on potyviral protein interaction maps did not include the 6K proteins (Guo *et al.*, 2001). In subsequent studies they were added to the map, but no interactions of these two proteins were detected (Kang *et al*., 2004; Shen *et al*., 2010). Lin *et al.* (2009), were the first who could observe interaction between 6K1 and 6K2 and other proteins, respectively. While 6K2 was shown to function as a membrane anchor for the replication complex in genome replication (Restrepo-Hartwig & Carrington, 1994; Schaad *et al*., 1997a) the function(s) or role of 6K1 is not well understood. It also localizes to the cell periphery, but it is in contrast to 6K2 no transmembrane protein (Hong *et al.*, 2007). As it was shown for PVA to be important for virus infectivity (Merits *et al.*, 2002) and interacts in a BiFC only with three proteins of the replication complex, 6K2, NIa and VPg, a participation in viral replication can be assumed.

Although this study delivers a very complex insight into the protein interaction network of PPV, a single protein of the ten tested proteins seemed to decrease the success of the BiFC system to present a reliable system for such investigations. While at least interactions of HC-Pro with itself, with CP and VPg were expected, astonishingly only one single interaction of PPV_HC-Pro with CI was observed. It was supposed that HC-Pro act as a homo-dimer in different stages of the virus infection cycle (Thornbury *et al.,*1985; Urcuqui-Inchima *et al.,*1999; Wang & Pirone*,*1999). Also for an interaction with CP, required for aphid-transmission, oligomers of at least two HC-Pro subunits are necessary (Ruiz-Ferrer *et al.*, 2005). Whereas other interactions of certain proteins may have been identified for one virus, but not for another one, all interaction studies ever performed with potyviral HC-Pro revealed at least a self-interaction of the

protein. So the question which was raised here again was whether the missing interaction of PPV-HC-Pro is due to methodological limitations of the BiFC system or to an species-dependent self-interaction of the protein. Therefore, different experiments were conducted with HC-Pro of PVY-N605 and two additional PPV strains, PPV-AT and PPV-BUL, also revealing negative results in the BiFC. The optimized BiFC system provides the possibility to generate N- as well as C-terminal mRFP fusions. Therefore, in a next step different constellations of HC-Pro fusions were tested to exclude that the steric arrangement of the fusion proteins does impede their association. However, also these analyses delivered negative results. Furthermore, since for TuMV_HC-Pro an interaction was observed not only with a YTH system but also in a very recently described BiFC approach clearly demonstrating a self-interaction of HC-Pro directly *in planta* (Zheng *et al.*, 2010), a putative methodological problem with the mRFP-based BiFC has to be taken into consideration. Therefore, parallel experiments with TuMV_HC-Pro and PPV_HC-Pro were conducted. TuMV_HC-Pro was clearly shown to interact with itself in the mRFP-based BiFC and also TuMV_HC-Pro as well as PPV_HC-Pro mutants revealed interaction. In summary, these data demonstrated the functionality of the system to detect HC-Pro self-interactions when occurring and support a species-specific or maybe even a strain-specific HC-Pro interaction. However, the reasons for differences in the interaction behavior of the HC-Pros are not clear. Since HC-Pro self-interaction seems to play a key role in other viruses, PPV_HC-Pro may otherwise fulfill its functions. It cannot be excluded, that also for PPV two HC-Pro subunits are needed to participate in single processes. But possibly this depends on some other proteins or special host factors, ions or even other viral factors, which function as some kind of bridging molecule. Therefore, an investigation of not only single protein interactions, but protein complexes, e.g. using a combination of BiFC and FRET, may be helpful and desirable.

Finally, in addition to the interaction studies BiFC technology allow to a certain extend to visualize the localization of protein-protein interactions in the living plant cells, which was exemplified for the self-interacting PPV proteins. The localization of CI, 6K2, VPg, NIa-Pro, NIb and CP interactions corresponded with expectations about the localization of these proteins. This clearly substantiated the power of this additional feature of the BiFC. While these six proteins have already been in focus of numerous studies, there is only little knowledge about the eleventh potyviral protein, the P3N-

PIPO. Wei *et al.* (2010b) performed different experiments with the TuMV_P3N-PIPO and revealed its involvement in cell-to-cell movement of TuMV. Mutational analysis of single proteins also involved in movement combined with subcellular co-localization studies and BiFC, enabled an optimal combination of different methods to get information on the function of the protein. Wei *et al.* (2010) were able to develop a model for the possible interplay of potyviral proteins during cell-to-cell movement through plasmodesmata. According to this P3N-PIPO, which was shown to localize to plasmodesmata, modulates the localization of CI-virion complexes to the plasmodesmata by an interaction between P3N-PIPO and CI. CI accumulates in typical conical structures (Rodríguez-Cerezo *et al.*, 1997; Roberts *et al*., 1998), which requires CI self-interaction, and probably recruit further virus particles for the transfer through plasmodesmata. The latter process probably requires an interaction between CI and CP. All these suggested interactions of proteins were clearly approved for the homologous PPV proteins in this work, which support the hypothesis of Wei *et al.* (2010b; schematic depictions of the model also presented in Niehl & Heinlein (2011)). Moreover, the results obtained in this study underline the hypothesis of Wei *et al.* (2010b) in that way, that neither a self-interaction of P3N-PIPO nor an interaction of CP and P3N-PIPO was detected in the BiFC assay. Therefore, some kind of 'bridging' between P3N-PIPO and CP by the CI may occur during the process of cell-to-cell movement. Another study also suggested that the P3N-PIPO of SMV is essential for the movement of SMV. Wen & Hajimorad (2010) performed mutational analysis with SMV_P3N-PIPO and monitored cell-to-cell movement of different mutants via GUS expression in soybean plants. The results of these experiments substantiate the hypothesis that P3N-PIPO is involved in cell-to-cell movment. In summary, the BiFC results of this work complement the results of other studies and emphasize the capability of BiFC in revealing the localization of interacting proteins as it has already been described by other groups (Citovsky *et al.*, 2006, 2008; Martin *et al.*, 2009). However, since exact subcellular localization of protein-protein interactions was not in the focus of this study, but should only be exemplarily demonstrated, the obtained results can be clearly extended and specified by future co-localization studies. Citovsky *et al.* (2008) provide an extensive overview on the possibilities of localization studies employing the BiFC in a wide range of plants. Thus, BiFC in combination with genetic methods and co-localization studies with labeled proteins and reference fluorecent markers, delivers a powerful tool for revealing

the functions of proteins and their participation in certain processes. BiFC technology therefore provides a feature, which YTH systems definitely cannot afford. This again underlines the limitations of the yeast technology.

However, regardless of how interaction studies are performed, with YTH systems, BiFC or any other method depending on protein fusions, it will not be possible to make exact statements about the real occurrence of interactions *in planta*. It cannot be excluded, that due to the fusion of proteins of interest to a fluorescent protein, the behavior of these proteins is altered. The methods can only deliver a first insight into or a hint for the interaction networks *in planta*. Nevertheless, the BiFC still seems to be a more reliable method to help in that than the YTH technology.

Taken together, the investigation of plum pox potyviral protein interactions *in planta* was highlighted in the work presented here and the discrepancies between BiFC and YTH systems were discussed. On the one hand it is suggested that BiFC investigations more resemble more natural conditions than yeast assays and found interactions in BiFC approaches rather represent the real occurrence in the plant. Employing the BiFC system more protein interactions were found for PPV than ever reported for any other potyvirus in a yeast system. On the other hand, for investigation of potyviral protein interactions - and this will be also true for viruses of other genera - it has to be considered that direct comparisons between interactions of different viruses seem to be critical, as seen for PPV_CP and PPV_HC-Pro. While the basic parts of the viral life cycle are very likely similar between single viruses of a genus and homologous proteins involved in the same processes, it is possible that protein interactions might differ between the viruses (maybe even in different hosts).

This work has delivered a suitable and practicable method for the study of plant viral protein-protein interactions *in planta*. Beyond an application in plant viral research it may be as well applicable for protein interaction studies of other viruses or organisms. It therefore may help many plant virologists as well as other research groups to get insight into the protein networks of viruses and organisms. Moreover, this work has contributed to a better understanding of the PPV protein interactions probably occurring during the infection cycle. It delivers single pieces of a puzzle, but further studies are necessary and will help to clarify the participation of PPV proteins in the infection cycle.

5 Outlook

Results obtained in this study on the one hand give first hints for the complex interplay of PPV proteins during the infection cycle. On the other hand, due to the ubiquitous question whether the found interactions represent the real occurrence in the plant, further research is needed.

As PPV_HC-Pro could not be shown to self-interact *in planta*, the possibility that a further protein may be needed for an interaction was suggested. Also for all other protein combinations where no interactions were detected in the BiFC an interference of other proteins by some kind of 'bridging' cannot be excluded. Therefore, protein-protein interaction studies in PPV infected plants would be helpful and desirable to get information on possible requirement of further proteins in certain protein interactions. A further possibility to achieve this aim is a direct investigation of protein complexes. Since the use of BiFC is restricted to binary protein-protein interactions, a combination of BiFC and FRET or BRET may help in identifying multimeric protein complexes.

By such combined methods there would also be the possibility to investigate the relevance of host factors for viral protein interactions. As mentioned for, e.g., VPg and NIb, not only interactions between viral proteins but also between viral proteins an host factors play an important rule in viral processes. Therefore, screening of plant protein libraries against viral proteins would be helpful to get information about this and to get a complete overview on interactions occurring during the infection cycle. However, this needs the help of further methods as BiFC does not allow a screening of libraries.

Further analysis of PPV protein interactions could include structural analyses. Structural information on proteins might help in the validation whether an interaction is a real or false positive/negative interaction. The main consideration aside from technical feasibility, is to get clear predictions about the occurrence of interactions.

N. benthamiana represents an experimental host for many potyviruses and was used throughout this work. It is easily manageable and allows fast and straightforward investigation of protein-protein interactions by BiFC. Nevertheless, it is no natural host of potyviruses and therefore further research will be needed in host plants like peach or apricot trees. However, since the experimental procedure including agroinoculation in woody plants is not that easy as in herbaceous plants, such experiments would include

the improvement of inoculation techniques.

Finally, in addition to interaction studies, the BiFC technology enables visualization of interactions at subcellular level. As shown for P3N-PIPO co-localization studies can help to reveal the involvement of viral proteins in certain processes. So localization of certain protein-protein interactions utilizing special cellular fluorescence markers, revealing of protein complexes as well as structural analyses of proteins and protein-interactions might shed new light on the network and exact mechanisms of the processes during viral infection cycle.

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ACKNOWLEDGEMENTS

Als erstes - und ganz besonders - bedanke ich mich bei Prof. Dr. Edgar Maiß; zum einen für die Bereitstellung des Themas, aber vor allem für die fantastische Betreuung während der gesamten Arbeit. Mit sehr viel Geduld, steter Diskussionsbereitschaft und vor allem einem unerschöpflichen Enthusiasmus war er mir ein hervorragender Lehrmeister.

Priv.-Doz. Dr. Christina Wege möchte ich an dieser Stelle ganz herzlich für die Übernahme des Korreferats danken.

Bei Herrn Prof. Dr. Michael Poehling bedanke ich mich für die Möglichkeit zur Durchführung dieser Arbeit am Institut für Pflanzenkrankheiten und Pflanzenschutz.

Frau Dr. Gisela Grunewaldt-Stöcker danke ich herzlich für die Einführung in die konfokale Mikroskopie und ihre stete Unterstützung bei allen Fragen rund um die Mikroskopie.

Besonders möchte ich mich an dieser Stelle bei Yvonne Koleczek bedanken: für die großartige Hilfe im Labor, die vielen netten Gespräche und die moralische Unterstützung – einfach für eine schöne Zeit. Auch Jutta Zimmermann, Till Lesker und Dominik Klinkenbuß, sowie allen anderen jetzigen und ehemaligen Mitgliedern der AG Maiß gebührt mein Dank für eine fantastische Arbeitsatmosphäre mit netten Gesprächen und steter Hilfe bei allen kleinen und großen Problemen. Dieser Dank gilt auch allen weiteren Mitarbeitern des Instituts.

Ein großes Dankeschön geht auch an Christiane von den Berg für viele tolle Gespräche, ihren unermüdlichen Ideenreichtum und ihre enorme Hilfsbereitschaft.

Ganz besonderer Dank gilt jedoch meiner ganzen Familie. Meinen Eltern danke ich von Herzen dafür, dass sie mich in all der Zeit stets unterstützt haben und immer an mich geglaubt haben. Meine Schwester war mir sowohl bei fachlichen Fragen als auch als ‘Motivatorin’ und vor allem als Freundin eine unersetzliche Hilfe. Vielen Dank Euch allen!

Schließlich möchte ich mich nun noch bei einem ganz besonderen Menschen bedanken. Für seine liebevolle Unterstützung in all der Zeit, seine unglaubliche Geduld, seine aufmunternden Worte, aber auch für die Zeit fernab des Doktorandendaseins danke ich meinem Mann Heiko von ganzem Herzen.

ERKLÄRUNG ZUR DISSERTATION

gemäß §6(1) der Promotionsordnung der Naturwissenschaftlichen Fakultät der Gottfried Wilhelm Leibniz Universität Hannover für die Promotion zum Dr. rer. nat.

Hierdurch erkläre ich, dass ich meine Dissertation mit dem Titel:

'Optimization and implementation of a bimolecular fluorescence complementation (BiFC) system for the detection of plum pox potyviral protein-protein interactions *in planta'*

selbständig verfasst und die benutzten Hilfsmittel und Quellen sowie gegebenenfalls die zu Hilfeleistungen herangezogenen Institutionen vollständig angegeben habe.

Die Dissertation wurde nicht schon als Masterarbeit, Diplomarbeit oder andere Prüfungsarbeit verwendet.

Hannover, Mai 2011

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(Unterschrift)

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