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

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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, Nla-Pro, Nlb 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.

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 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 a single, long ORF that is translated into a polyprotein precursor of approximately 350 kDa. This precursor is processed by three virus-encoded proteases, which release ten mature viral proteins: the P1 protein; helper component–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 (Nla), with the N-terminal VPg and a C-terminal protease (Nla-Pro); nuclear inclusion protein b (Nlb); 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 (Urcuci-Inchima et al., 2001); for example, 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. The identification and investigation of protein–protein interactions comprise an important step in understanding the virus infection cycle and the interplay between virus and host. Several methods have been developed to identify and examine protein–protein interactions. In addition to different in vitro methods (Phizicky...
protein are brought together by the association of inter-
N- and C-terminal non-fluorescent halves of a fluorescent 
based on the formation of a fluorescent complex when the 

et al.

under natural conditions is useful and desirable.

studies, but subsequent examination with an 

1999; Lo´ pez 

G7H and SMV-P (Kang 

2009) and strains of soybean mosaic virus (SMV), SMV-
papaya ringspot virus strain P (PRSV-P) (Shen 
et al.

PPV, representing the first BiFC-based potyviral interaction 

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 

RESULTS AND DISCUSSION

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 analyse 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, 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, 1–221, 98–315, 
98–221 and 222–315, respectively (Fig. 2a), and the capability 
of the full-length CP (CPfull) and the mutants to interact 
with themselves and each other was tested. Therefore, N. benthamiana plants were infiltrated with mixtures of three 
Agrobacterium tumefaciens cultures harbouring the expres-

411x506], 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 strains of soybean mosaic virus (SMV), SMV-
97, 1–221, 98–315, 
98–221 and 222–315, respectively (Fig. 2a), and the capability 
of the full-length CP (CPfull) and the mutants to interact 
with themselves and each other was tested. Therefore, N. benthamiana plants were infiltrated with mixtures of three 
Agrobacterium tumefaciens cultures harbouring the expres-
sion plasmids pCB:COI-mRFPN, pCB:COI-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 post-infiltration (p.i.) by confocal laser-
scanning microscopy (CLSM; Fig. 2b–e).

Co-expression of CPfull–mRFP, F1–mRFP and F2–mRFP 
fragments revealed red fluorescence in the cytoplasm of 
epidermal cells (Fig. 2b–d), representing self-interaction of 
CPfull, F1 and F2 mutants, respectively. However, after co-
infiltration 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 the CPfull, 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 infiltrated leaves. In subsequent studies, the F3–mRFP and CPfull–mRFP fragments served as a non-interacting and an interacting control, respectively.

PPV_CP interaction studies suggested that the F1 and F2 CP fragments contain interacting protein domains and that 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 CP in a YTH approach. Studies on the assembly of TEV, johnsongrass mosaic virus and PPV particles in Escherichia coli and in planta (Dolja et al., 1991, 1994; Jagadish et al., 1993; Jacquet et al., 1998; Varrelmann & Maiss, 2000; Voloudakis et al., 2004) have revealed two highly conserved amino acid 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 the core nor the C-terminal region was determined to be necessary for the physical self-interaction of the CPs in SMV-G7H (Kang et al., 2006) and PPV. This may indicate that the aforementioned amino acid motifs are not essential for the physical interaction of single CP subunits, but are involved in building particle-like structures; the domains that are important for self-interaction do not seem to be conserved among potyviruses. Moreover, it is very likely that the results of a BiFC assay more closely resemble the real conditions in planta than YTH approaches. To determine which amino acid motifs are necessary or sufficient for CP self-interactions, further detailed investigations with potyviral CPs are needed.

**Interaction matrix of PPV proteins**

An interaction matrix of ten proteins from PPV was generated using plasmids pBIN19 : COI-mRFPN and pBIN19 : COI-mRFPC to create N-terminal mRFP fusion proteins. Distinct full-length PPV cistrons 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 infiltrated N. benthamiana leaves were prepared at 3 days p.i., and immunoblot analyses were performed using anti-c-Myc and anti-haemagglutinin (HA) antibodies to detect the mRFPN and mRFPC fusion proteins, respectively. All of the PPV proteins, including P3N-PIPO, were expressed at detectable levels, and the estimated molecular masses were in accordance with the calculated ones (Fig. 3). Additional protein bands of approximately 20 and 50 kDa were observed in all of the samples on the immunoblots 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 (anti-c-Myc and anti-HA) for P1 and with the anti-c-Myc antibodies for some other proteins, and may represent prematurely terminated proteins or proteolytic degradation products occurring during protein extraction. In the cases of P1 and NIa-Pro, these bands presumably represent fragments from proteaseinase activity.

The fluorescence of mRFP was visualized at 3 days p.i. in single leaf discs of infiltrated 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 Table 1. Red fluorescence, representing interaction between the tested proteins, was detected for 52 protein combinations; among these, we observed the self-interaction of

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**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 cistrons are indicated. P3N-PIPO and PIPO are marked by black bars. Below the genome organization is a map of the CP deletion mutants. Full-length CP (CPfull) was divided into three fragments by PCR mutagenesis, and five mutants were generated. CPfull 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 CPfull and its mutants were tested by BiFC. CLSM images of the epidermal leaf cells co-infiltrated with mixtures of agrobacteria harbouring the expression plasmids pCB:CPfull-mRFPN and -mRFPC (b), pCB:F1-mRFPN and -mRFPC (c), pCB:F2-mRFPN and -mRFPC (d) and pCB:F3-mRFPN and -mRFPC (e). Bars, 50 μm. (f) Schematic overview of the tested CP mutant combinations: +, fluorescence detected; −, fluorescence not detected.
CI, 6K2, VPg, Nla-Pro, Nilb and CP (Table 1). In addition, CI interacted with all of the other PPV proteins. VPg, Nla-Pro and Nilb also interacted with at least six other proteins, whereas only a few interactions were observed for P1, HC-Pro and P3. For the 6K proteins, 6K2 self-interaction and a 6K1–6K2 interaction were demonstrated for the first time.

In contrast to all previously reported interaction maps, 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, a more complex interaction matrix would be expected, as is described in the present study and was reported by Lin et al. (2009).

Table 1. Interactions of different PPV proteins

<table>
<thead>
<tr>
<th>mRFPN fusion</th>
<th>mRFPC fusion</th>
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</thead>
<tbody>
<tr>
<td>P1 HC-Pro</td>
<td>P3 6K1 CI</td>
</tr>
<tr>
<td>VPg Nla-Pro</td>
<td>Nilb CP</td>
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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 mAbs and the mRFPC fusion proteins with anti-HA mAbs in the immunoblot analyses of N. benthamiana leaf material at 3 days p.i. The analysed fusion proteins are listed. The molecular masses of the detected proteins were determined using the Spectra Multicolor Broad Range Protein Ladder or the Page Ruler Prestained Protein Ladder Plus (Fermentas). Arrowheads show the full-length fusion proteins at their calculated sizes, Myc–linker–mRFPN/HA–linker–mRFPC fusions (kDa): P1, 54.5/43.0; HC-Pro, 72.3/60.8; P3, 60.0/48.5; 6K1, 25.0/13.5; CI, 92.3/80.8; 6K2, 25.1/13.6; VPg, 41.5/30.0; Nla-Pro, 46.7/35.2; Nilb, 78.4/67.0; CP, 55.4/43.9; P3N-PIPO, 46.9/35.4.

a CI self-interaction and interactions with all of the 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 behaviour, 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.

Except 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, Nla-Pro and CP was observed only when P1 was fused to mRFPN and the other proteins were 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, Nla-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 favourable protein folding, which may also be true in the 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 the proteins of interest.

To exclude the possibility that the 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-COI and pCB:mRFPC-COI, 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.

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, Nla and Nlb) that self-interacted, and the self-interaction of PPV_CP is demonstrated in Fig. 2(b). 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., 2010a). However, co-localization studies were not conducted and, therefore, an exact localization of the proteins was not possible. The red fluorescence of the VPg self-interaction was observed predominantly in the nucleus of the cells (Fig. 4b), with an accumulation in the nucleolus, reflecting the nuclear localization of VPg, as has been demonstrated for different potyviruses (Schaad et al., 1996; Beauchemin et al., 2007). An accumulation of fluorescence in nuclei and in the cytoplasm of epidermal cells was observed after the co-expression of Nla–mRFP fusions (Fig. 4c), and fluorescence derived from Nlb self-interaction was detected in small aggregates along the cell walls (Fig. 4d). For 6K2, self-interaction fluorescence in vesicular structures was observed (Fig. 4e), the autofluorescence of chlorophyll in chloroplasts was clearly demonstrated, and 6K2 was shown to accumulate in the chloroplasts (Fig. 4f), as reported by Wei et al. (2010b).

Chung et al. (2008) described an additional ORF (PIPO) embedded in the P3 cistron of turnip mosaic virus (TuMV) and other potyviruses, encoding a P3N-PIPO fusion protein. Previously, an interaction of P3N-PIPO and CI was identified by a BiFC assay for TuMV and was proposed to be essential for cell-to-cell movement (Wei et al., 2010a). 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. The co-expression of P3N-PIPO and CI resulted in fluorescence complementation (Fig. 5a, 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, this finding requires confirmation by the use of appropriate localization markers in additional studies. The CP and CI interaction was analysed further (Fig. 5c, 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. (2010a), and 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.

Our 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. Some interactions are easily localized in the nucleus/nucleolus (e.g. VPg) or the chloroplasts (e.g. 6K2). However, the confirmation of other subcellular localizations requires supplementation with fluorescent reference markers, which are co-expressed with the fusion proteins (Citovsky et al., 2008).

In our study, new and known potyviral protein interactions were demonstrated with the BiFC system. Surprisingly, neither an interaction between 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
Fig. 4. Self-interactions of the PPV proteins. BiFC of mRFP in N. benthamiana epidermal cells at 3 days p.i. CLSM images for the mRFP fluorescence and merged pictures with the transmitted light mode of cells co-infiltrated with pBIN19: Cl-mRFPN and -mRFPC (a), pBIN19: VPg-mRFPN and -mRFPC (b), pBIN19: Nla-Pro-mRFPN and -mRFPC (c), pBIN19: Nb-mRFPN and -mRFPC (d) and pBIN19: 6K2-mRFPN and -mRFPC (e). Bars, 50 μm. For 6K2 self-interaction, the mRFP domain was excited at 543 nm and the emitted light was captured at 600–610 nm. The autofluorescence of chlorophyll was visualized in parallel by excitation at 488 nm and the detection of fluorescence at 690–740 nm (f) to demonstrate 6K2 localization in the chloroplasts. Bars, 25 μm.
PPV_HC-Pro in our in planta BiFC. Therefore, more detailed experiments with PPV_HC-Pro were conducted.

**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. (2011) analysed TuMV_HC-Pro with regard to self-interaction and mapped protein 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. (2011), 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 analyse the self-interaction of the full-length proteins and their mutants, N. benthamiana plants were infiltrated, and the fluorescence was recorded at 3 days p.i. 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-infiltration of the TuMV_HC-ProN–mRFP fragments and the TuMV_HC-ProC–mRFP fragments revealed fluorescence (Fig. 6c, 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, 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, and it has been proposed that HC-Pro acts as a homodimer 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 (Ruiz-Ferrer et al., 2005). All of these studies have led to the conclusions that HC-Pro acts as a 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. Furthermore, experiments with the HC-Pros of two additional PPV strains [PPV-AT and a Bulgarian strain of PPV (DSMZ, PV-0212)] and PVY (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., 2011) 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 interac-
performed using Phusion Flash Master Mix (Finnzymes) and the P3N-PIPO and pCB : mRFPC-P3N-PIPO. PCR mutagenesis was performed with Phusion Flash Master Mix using the P3Y2H_s and PIPO as primers (Supplementary Table S1), which introduced BamHI and SalI sites. The generated fragment was digested with BamHI/SalI and used to replace PPV_CP in pCB::mRFPC and pCB::TuMV_HC-Pro-mRFPC. The resulting plasmids were designated pCB::TuMV_HC-Pro-mRFPC and pCB::TuMV_HC-Pro.mRFPC.

These two plasmids and pCB::PPV_HC-Pro-mRFPC and pCB::PPV_HC-Pro-mRFPC were used as templates for the PCR mutagenesis of the TuMV_HC-Pro and PPV_HC-Pro constructs. Plasmids encoding the mRFP fusions with the N-terminal regions of the HC-Pro (aa 1–99) were generated by PCR mutagenesis with Phusion Flash Master Mix using primers PPV_HC-Pro_N1 and HC-Pro_N2 with PPV_HC-Pro, and primers TuMV_HC-Pro_N1 and HC-Pro_N2 (Supplementary Table S1) with TuMV_HC-Pro. The resulting plasmids were designated pCB::PPV_HC-Pro-mRFPC and -mRFPC, and pCB::TuMV_HC-Pro-mRFPC and -mRFPC. For the generation of plasmids encoding mRFP fusions with the C-terminal regions of the HC-Pro (aa 100–458), PCR mutagenesis was performed with the primers HC-Pro_C1 and TuMV_HC-Pro_C2 with PPV_HC-Pro, and the primers HC-Pro_C1 and TuMV_HC-Pro_C2 (Supplementary Table S1) with TuMV_HC-Pro, to yield the pCB::PPV_HC-ProC-mRFPC and -mRFPC, and pCB::TuMV_HC-ProC-mRFPC and -mRFPC plasmids.

**Methods**

### Construction of the expression plasmids for BifC.

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

### Construction of the PPV_CP plasmids.

Full-length CP, derived from a PPV-NAT full-length clone (Mai et al., 1992), was PCR-amplified with primers CPY2H_s and CPY2H_as (Supplementary Table S1) with TuMV_HC-Pro. The PCR-amplified CP fragment was digested with BamHI/FseI and ligated into binary plasmids digested with BamHI/FseI. The resulting plasmids were designated pBat_TL-smRFP_N and pBat_TL-smRFP_C, and the working group of D. Priuer (University of Münster, Germany), especially B. Kommer, for providing the agrobacteria harbouring the pCH32 plasmid. We are also grateful to

**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 TuMV (DSMZ PV-0104)-infected N. benthamiana with RevertAid Premium Reverse Transcriptase (Fermentas) and Phusion Flash Master Mix using the TuMV_HC-Pro_s and TuMV_HC-Pro_as primers (Supplementary Table S1), which introduced BamHI and SalI sites. The generated fragment was digested with BamHI/SalI and used to replace PPV_CP in pCB::mRFPC and pCB::TuMV_HC-Pro-mRFPC. The resulting plasmids were designated pCB::TuMV_HC-Pro-mRFPC and pCB::TuMV_HC-Pro.mRFPC.

**Transcript protein expression in N. benthamiana leaf epidermal cells and CLSM.** Binary expression plasmids were electroporated into A. tumefaciens strain C58C1 (Deblaere et al., 1985) for the infiltration of N. benthamiana plants. Agrochemical cultures harbouring the BiFC plasmids and pHCh3 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 3 days at room temperature before single discs of infiltrated N. benthamiana leaves were assayed for fluorescence by CLSM using a Leica TCS SP2 confocal microscope. The mRFP domain was excited at 543 nm with the green neon laser, and the emitted light was captured at 600–610 nm under constant-recording conditions. The autofluorescence of chlorophyll was visualized by excitation at 488 nm with the argon/cyanter laser and detection of fluorescence at 690–740 nm. The images were captured digitally and processed using the Leica confocal software.

**Immunoblot analysis.** Expression of the protein fusions was verified in total leaf protein extracts using anti-HA- and anti-c-Myc-specific mAbs for detection. Protein extracts at 3 days p.i. were prepared from leaves expressing COI::mRFPN and COI::mRFPC fusions (modified after Berger et al. (1989)), Tricine/SDS-PAGE (Schagger and von Jagow, 1987), Western blotting (Towbin et al., 1979) and detection of luminescence was performed as described previously (Zilian & Maiss, 2011).

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