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 horticultural 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 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 (Urcuqui-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
& 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. However, this system bears limitations, including systematic false-negative and -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 analysed 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; See 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-G7H and SMV-P (Kang et al., 2004; Lin et al., 2009) have been generated. Although no complete interaction map of proteins from PPV has been developed thus far, López et al. (2001) analysed the CI protein with respect to its self-interaction and interaction with five other PPV proteins, giving a first, but incomplete, insight into the interactome of this virus.

Focusing on 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 behaviour 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 (Hu et al., 2002; Atmakuri et al., 2003; Hu & Kerppola, 2003; Walter et al., 2004; Blondel et al., 2005; Cole et al., 2007; Sung & Huh, 2007; 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.

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., 2010a). Moreover, we demonstrate 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).

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 expression plasmids pCB:CO1-mRFPN, pCB:CO1-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 proteinase 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, Nlb and CP (Table 1). In addition, CI interacted with all of the other PPV proteins. VPg, Nla-Pro and Nlb 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

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+, Interaction detected; -, no interaction visible. Self-interactions are boxed.

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:mRFPC-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 (Attreye & 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: CI-mRFPN and -mRFPC (a), pBIN19: VPg-mRFPN and -mRFPC (b), pBIN19: Nla-Pro-mRFPN and -mRFPC (c), pBIN19: Nlb-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–mRFPC 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–mRFPC 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),

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

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 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 100–458, respectively. (b–f) CLSM images for the mRFP fluorescence and merged pictures with the transmitted light mode of cells co-infiltrated with TuMV_HC-Pro-mRFPN and -mRFPC (b), TuMV_HC-ProN-mRFPN and -mRFPC (c), TuMV_HC-ProC-mRFPN and -mRFPC (d), PPV_HC-ProN-mRFPN and -mRFPC (e) and PPV_HC-ProC-mRFPN and -mRFPC (f). Bars, 50 μm.
tions 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 the 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.

**METHODS**

**Construction of the expression plasmids for BiFC.** The pBIN19: COI-mRFPN and -mRFPC, pCB: COI-mRFPN and -mRFPC and pCB: mRFPN-COI 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 (Maiass et al., 1992), was PCR-amplified with primers CPY2H_S and CPY2H_as (Supplementary Table S1, available in JGV Online) and introduced into pGEM-T Easy (Promega) to produce pGEM-T Easy_CP. The CP cistron was divided into five fragments by PCR mutagenesis using Phusion Flash DNA polymerase (Finnzymes). The F1, F3, F1_2 and F2_3 fragments of PPV_CP, encoding aa 1–97, 222–315, 1–221 and 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_CI/PPV_CC2, PPV_N1/PPV_NC2 and PPV_CC1/PPV_CC2 primer pairs (Supplementary Table S1), respectively. Plasmid pGEM-T Easy_F2, encoding aa 98–221 of PPV_CP, was generated by the PCR amplification of pGEM-T Easy_F2_3 with the PPV_N1/PPV_NC2 primer combination (Supplementary Table S1). Finally, CP_Sall and CP fragments F1, F1_2, F2, F2_3 and F3 were BamHI/XhoI-digested and ligated into binary plasmids digested with BamHI/Sall.

**Construction of the plasmids for PPV full-length protein interactions.** The PPV cistrons P1, HC-Pro, P3, 6K1, CI, 6K2, VPg, Nla-Pro and Nlb were PCR-amplified from the PV-NAT full-length clone using Phusion Flash DNA polymerase and specific primers (Supplementary Table S1) that introduced BamHI and XhoI sites (except for Nla-Pro: BglII/Sall). The PCR-amplified fragments were digested with BamHI/XhoI (except for Nla-Pro: BglII/Sall) and inserted into binary plasmids digested with BamHI/Sall (except for Nla-Pro: BglII/Sall), yielding the respective BiFC plasmids.

**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 P3Y2H_s and PIPO_P3N-PIPO primers (Supplementary Table S1), which introduced BamHI and SalI sites. The generated fragment was digested with BamHI/Sall and used to replace PPV_CP in pCB: mRFPN and pCB: mRFPC. The resulting plasmids were designated pCB: TuMV_HC-Pro-mRFPN and pCB: TuMV_HC-Pro-mRFPC.

**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_HCPro_s and TuMV_HCpro_as primers (Supplementary Table S1), which introduced BamHI and SalI sites. The generated fragment was digested with BamHI/Sall and used to replace PPV_CP in pCB: mRFPN and pCB: mRFPC. The resulting plasmids were designated pCB: TuMV_HC-Pro-mRFPN and pCB: TuMV_HC-Pro-mRFPC.

**Transient 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. Agrobacterial cultures harbouring 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 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/cyan 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 (Schägger and von Jagow, 1987), Western blotting (Towbin et al., 1979) and detection of luminescence were performed as described previously (Zilian & Maiss, 2011).

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