Towards a protein interaction map of potyviruses: protein interaction matrixes of two potyviruses based on the yeast two-hybrid system

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A map for the interactions of the major proteins from Potato virus A (PVA) and Pea seed-borne mosaic virus (PSbMV) (members of the genus Potyvirus, family Potyviridae) was generated using the yeast two-hybrid system (YTHS). Interactions were readily detected with five PVA protein combinations (HC–HC, HC–Cl, VPg–VPg, Nia–Nlb and CP–CP) and weak but reproducible interactions were detected for seven additional combinations (P1–Cl, P3–Nlb, NiaPro–Nlb, VPg–Nia, VPg–NiaPro, NiaPro–Nia and Nia–Nia). In PSbMV, readily detectable interactions were found in five protein combinations (HC–HC, VPg–VPg, VPg–Nia, Nia–Nia and Nia–Nlb) and weaker but reproducible interactions were detected for three additional combinations (P3–Nia, Nia–NiaPro and CP–CP). The self-interactions of HC, VPg, Nia and CP and the interactions of VPg–Nia, Nia–NiaPro and Nia–Nlb were, therefore, common for the two potyviruses. The multiple protein interactions revealed in this study shed light on the co-ordinated functions of potyviral proteins involved in virus movement and replication.

Interactions between proteins play pivotal roles in many processes during the virus infection cycle; for example, in the formation of virus replication complexes, assembly of virions, virus movement between cells and virus transmission between plants by vectors. Analysis of protein–protein interactions is crucial for understanding protein functions and the molecular mechanisms underlying biological processes. The classical and more recent methods for detecting protein interactions have been reviewed previously (Phizicky & Fields, 1995; Mendelsohn & Brent, 1999; Vidal & Legrain, 1999). Among these, the yeast two-hybrid system (YTHS) (Fields & Song, 1989) represents a rapid and sensitive method for identifying protein–protein interactions.

YTHS has been used extensively to detect interactions between proteins from many different organisms. The results show a high correlation with affinities that are determined by biochemical methods (Estojak et al., 1995). Using YTHS, protein-linkage maps have been established for Escherichia coli bacteriophage T7 (Bartel et al., 1996), Saccharomyces cerevisiae RNA-splicing machinery (Fromont-Racine et al., 1997) and poliovirus P2 and P3 proteins (Cuconati et al., 1998; Xiang et al., 1998). The technique has also been used to explore the entire protein–protein interaction network within yeast cells (Ito et al., 2000; Uetz et al., 2000).

Potyviruses possess a single-stranded, positive-sense RNA genome of about 10 kb. The genomic RNA is translated into a large polyprotein that is subsequently processed by three virus-encoded proteinases to yield mature functional proteins P1, HC, P3, 6K1, CI, 6K2, VPg, NiaPro, Nlb and CP (Riechmann et al., 1992). A remarkable feature of potyviruses, compared with other plant RNA viruses, is that most potyviral proteins are multifunctional and several of them participate in both genome replication and virus movement (Revers et al., 1999; Carrington et al., 1998), which implies that at least some functions rely on viral protein–protein interactions.

Some interactions between potyviral proteins have been studied using YTHS (Hong et al., 1995; Li et al., 1997; Merits et al., 1999; Guo et al., 1999; Urcuqui-Inchima et al., 1999). However, interactions between all of the proteins from a single potyvirus have not been studied. The aim of this study was to generate a YTHS-based profile of potyviral protein interaction networks through a comprehensive analysis of interactions among all the major proteins from two potyviruses, Potato virus A (PVA) and Pea seed-borne mosaic virus (PSbMV), which are not closely related (Berger et al., 1997) and naturally infect species of different plant families (Solanaceae and Fabaceae, respectively), were chosen for this study; this choice was also favoured by the fact that full-length cDNAs of their genomes were available.

YTHS (Hollenberg et al., 1995) and the LexA system (Clontech) were used for the study of PVA and PSbMV.
Table 1. Protein–protein interactions of Potato virus A (PVA)

The yeast host strain L40 was used to determine protein–protein interactions (Hollenberg et al., 1995). The DNA-binding domain vector pLexA, with a TRPI selection marker derived from pBTM116 (Hollenberg et al., 1995), was used throughout. An activation domain (AD) vector, pVP16, with a LEU2 selection marker, was used for most constructs, except P3 and NiB, which were cloned into a GAL4-based AD vector pGAD424 (Clontech). +++, Colonies turned blue in < 2 h in filter assays and the relative β-galactosidase activity was > 50 units; ++, colonies turned blue after 2–6 h in filter assays and the relative β-galactosidase activity was 3–50 units; +, colonies turned blue after 6–12 h in filter assays and the relative β-galactosidase activity in liquid assay was not quantifiable; ±, few colonies grew on histidine-deficient plates and weak and inconsistent colour was noted after overnight incubation; –, very few or no colonies appeared on growth selection medium and colonies from transformant selection medium did not turn blue after overnight incubation.

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kindly provided by E. Johansen) using Expand High-Fidelity DNA polymerase (Boehringer Mannheim). PCR fragments were initially cloned into pGEM-T (Promega) and subsequently cloned into yeast fusion vectors (Tables 1, 2). All PCR clones and fusion junction sequences in the YTHS vectors were verified by sequencing.

To construct an interaction map for all of the major proteins for each of the two potyviruses, pairs of plasmids, a BD-construct and an AD-construct, each carrying one viral gene sequence, were co-transformed into yeast cells by the lithium acetate method (Schiestl & Gietz, 1989). Human lamin C protein, which does not form complexes or interact with most other proteins (Bartel et al., 1993), was used as a negative control in the matrix assays. The expression of fusion proteins was assessed by Western blot analysis (Guo et al., 1999). As shown previously by Merits et al. (1999), all PVA proteins were correctly expressed in yeast cells at detectable levels. This was also the case with PSbMV proteins (Fig. 1), except for P1 and Cl, which were not detected in several independent cloning and expression experiments, suggesting that they were subject to quick turnover or instability in yeast cells. Expression of Nla from both viruses also resulted in detectable amounts of VPg and NlaPro, which indicates that Nla is proteolytically active in yeast cells (Fig. 1). Protein bands that were smaller than the expected full-length proteins were also observed for HC, VPg, NlaPro and Nla fusion proteins. These may represent prematurely terminated polypeptides or proteolytic degradation products (Fig. 1). Since AD-constructs
than those in the other direction. more favourable protein folding or exposure of binding sites disparity implies that protein fusions in one direction may have interaction between BD–NIb and AD–NIa (Tables 1, 2). Such detected only when these proteins were expressed in fusion example, interactions between P3 and NIa from PSbMV were specifically by the viral protein parts in the BD- and AD-background level. The interactions were mediated, therefore, control), the activities of the reporter genes were reduced to mentioned above was replaced with human lamin C (negative al.

expression of their proteins is not shown.

Expression of the $\beta$-galactosidase reporter gene was evaluated qualitatively by filter assays (Merits et al., 1999) and quantitatively by liquid assays (O'Reilly et al., 1995). Interactions were readily detected with PVA protein combinations HC–HC, HC–CI, VPg–VPg, Nla–Nlb and CP–CP and weak but reproducible interactions were detected for P1–CI, P3–Nlb, NlaPro–Nla, NlaPro–Nlb, VPg–Nla, VPg–NlaPro and Nla–Nla (Table 1). Of these interactions, HC–HC, P1–CI, P3–Nlb and CP–CP were detected for HC–HC, P1–CI, P3–Nlb and CP–CP and weak but reproducible interactions were detected for P1–CI, P3–Nlb, NlaPro–Nla, NlaPro–Nlb, VPg–Nla, VPg–NlaPro and Nla–Nla (Table 1). Of these interactions, HC–HC, P1–CI, P3–Nlb and CP–CP have been described previously (Guo et al., 1999; Merits et al., 1999). Interactions between PSbMV proteins, which have not been studied previously, were readily detected with the combinations HC–HC, VPg–VPg, VPg–Nla, Nla–Nla and Nla–Nlb (Table 2) and weak but reproducible interactions were detected with P3–Nla, NlaPro–Nla and CP–CP (Table 2). Hence, the self-interactions of HC, VPg, Nla and CP and the interactions of VPg–Nla, Nla–NlaPro and Nla–Nlb were common for the two potyviruses.

If one interacting partner in any of the combinations mentioned above was replaced with human lamin C (negative control), the activities of the reporter genes were reduced to background level. The interactions were mediated, therefore, specifically by the viral protein parts in the BD- and AD-fusions. Some interactions also showed directionality. For example, interactions between P3 and Nla from PSbMV were detected only when these proteins were expressed in fusion with BD and AD, respectively (Table 2). Similarly, the interaction between BD–Nlb and AD–Nla was stronger than that between BD–Nla and AD–Nlb (Tables 1, 2). Such disparity implies that protein fusions in one direction may have more favourable protein folding or exposure of binding sites than those in the other direction.

The relative strengths of different interactions were compared by quantitative assays of $\beta$-galactosidase activity. In all experiments, $\beta$-galactosidase activity in liquid assays correlated with colour development in filter assays. Colonies harbouring interacting partners, which developed a visible blue colour between 1 and 2 h in filter assays, all produced more than 50 relative units of $\beta$-galactosidase activity (Tables 1, 2). Colonies that developed a visible colour by 6 h in filter assays gave between 3 and 50 relative units of $\beta$-galactosidase activity in liquid assay (Tables 1, 2). $\beta$-Galactosidase activity could not be detected easily in liquid assays in the colonies that developed a visible colour after overnight incubation; however, after prolonged incubation (12 h), visible colour was at least two times higher than that in negative controls, including the human lamin C fusions (Tables 1, 2).

An interaction between CI and HC proteins has not been reported previously for any potyvirus, except, very recently, for Wheat streak mosaic virus (WSMV), which is a member of the family Potyviridae, genus Tritimovirus (Choi et al., 2000). The interaction between HC and CI may be relevant to their co-ordinated functions in virus cell-to-cell movement (Cronin et al., 1995; Kasschau et al., 1997; Rojas et al., 1997; Carrington et al., 1998). Similarly, an interaction between P1 and CI may be relevant to the involvement of these proteins in virus movement and replication mediated by putative protein complexes (Merits et al., 1999). This is supported by the colocalization of P1 and CI in cells infected by Potato virus Y (Arbatova et al., 1998).

The VPg–VPg interaction reported here is a novel observation for PSbMV and is supported by the more extensive studies carried out on the self-interaction of PVA VPg (Oreutebaria et al., 2001). The potyviral VPg protein mediates aggregation of viral RNA (Luciano et al., 1991) and

### Table 2. Protein–protein interactions of Pea seed-borne mosaic virus (PSbMV)

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Criteria were similar to those used in Table 1, except that leucine-deficient plates were used for growth selection. The yeast host strain EGY48 (Clontech) was used. The DNA-binding domain vector pLexA, with a HIS3 selection marker (Clontech), and pB42AD, with a TRP1 selection marker (Clontech), were used for all experiments. P1 and CI proteins from PSbMV were not detectable in yeast cells and, thus, results from interactions involving P1 and CI were inconclusive and are indicated with asterisks.
the VPg–VPg interaction might facilitate recruitment of virus replication and/or translation complexes to the right position on the viral genomic RNA.

The physical interaction between NLb and Nla in yeast cells, shown in this study for both PVA and PSbMV (Tables 1, 2), was detected previously with two other potyviruses, **Tobacco vein mottling virus** (TVMV) and **Tobacco etch virus** (TEV) (Hong et al., 1995; Li et al., 1997). However, previous studies had suggested that the domain of Nla which is involved in the interaction with NLb might be different among potyviruses, since the N-terminal domain of VPg interacted with NLb in TVMV (Hong et al., 1995, Fellers et al., 1998), whereas the C-terminal proteinase domain (NlaPro) interacted with NLb in TEV (Li et al., 1997; Daros et al., 1999). In this study, elevation of reporter gene activity occurred upon co-expression of NlaPro and NLb, in contrast to co-expression of VPg and NLb of PVA (Table 1), suggesting that the Nla–NLb interaction may be more likely to be mediated by the proteinase domain of Nla from PVA, as was shown previously with TEV (Li et al., 1997). In this and previous studies (Merits et al., 1999), it has been observed that Nla expressed in yeast undergoes partial autocleavage into VPg and NlaPro and results in fusion proteins containing either VPg or full-length Nla in yeast cells. VPg interacts with itself and, consequently, with Nla, which contains the VPg domain (Tables 1, 2). These results might imply that the Nla–NLb interaction is mediated by a dimer formed by VPg and full-length Nla in which the NlaPro domain is in contact with NLb. This model is also consistent with a previous report that showed that certain mutations in the VPg domain abolish the interaction between Nla and NLb (Hong et al., 1995).

Interactions among the putative components of the potyvirus replication complex (NLb, Nla and VPg) shed light on their co-ordinated functions in virus replication. Nevertheless, no detectable interactions were observed between CI and other components of the replication complex, suggesting that CI might be recruited to the replication complex by its RNA-binding properties (Merits et al., 1998).

Some of the protein–protein interactions identified in this study have been observed previously with other potyviruses (Hong et al., 1995; Li et al., 1997; Urcuqui-Inchima et al., 1999). Very recently, a protein–protein interaction study was published on WSMV, a tritimovirus with genome organization similar to potyviruses (Choi et al., 2000). The study was carried out by YTHS expressing random viral cDNAs and this resulted in more interactions between protein domains than those obtained using full-length P1, HC, P3 and CI. It appears that proteins derived from random cDNAs can generate more interactions that are not influenced by global folding. Nevertheless, without structural knowledge of individual proteins, it is difficult to judge which kind of interaction reflects the real situation.

YTHS has emerged as a powerful method for detecting protein interactions (Fields & Song, 1989). A large number of protein–protein interactions have been identified from various organisms by YTHS in the last 10 years, contributing greatly to functional studies of proteins (Vidal & Legrain, 1999). Nevertheless, YTHS has limitations for studying protein–protein interactions, as do other methods, such as, for example, in vitro binding assays (Merits et al., 1999). While the YTHS results are tantalizing, they are not necessarily in agreement with in vitro binding data (Merits et al., 1999) and confirmation in an in planta system is desirable. In general, the knowledge obtained from YTHS may serve as a basis for further characterization of potyviral protein–protein interactions and their biological functions.

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References


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