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# Multiple Interactions among Proteins Encoded by the Mite-Transmitted Wheat Streak Mosaic Tritimovirus

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The genome organization of the mite-transmitted wheat streak mosaic virus (WSMV) appears to parallel that of members of the Potyviridae with monopartite genomes, but there are substantial amino acid dissimilarities with other potyviral polyproteins. To initiate studies on the functions of WSMV-encoded proteins, a protein interaction map was generated using a yeast two-hybrid system. Because the pathway of proteolytic maturation of the WSMV polyprotein has not been experimentally determined, random libraries of WSMV cDNA were made both in DNA-binding domain and activation domain plasmid vectors and introduced into yeast. Sequence analysis of multiple interacting pairs revealed that interactions largely occurred between domains within two groups of proteins. The first involved interactions among nuclear inclusion protein a, nuclear inclusion protein b, and coat protein (CP), and the second involved helper component-proteinase (HC-Pro) and cylindrical inclusion protein (CI). Further immunoblot and deletion mapping analyses of the interactions suggest that subdomains of CI, HC-Pro, and P1 interact with one another. The two-hybrid assay was then performed using full-length genes of CI, HC-Pro, P1, P3, and CP, but no heterologous interactions were detected. *In vitro* binding assay using glutathione-*S*-transferase fusion proteins and *in vitro* translation products, however, revealed mutual interactions among CI, HC-Pro, P1, and P3. The failure to detect interactions between full-length proteins by the two-hybrid assay might be due to adverse effects of expression of viral proteins in yeast cells. The capacity to participate in multiple homomeric and heteromeric molecular interactions is consistent with the pleiotropic nature of many potyviral gene mutants and suggests mechanisms for regulation of various viral processes via a network of viral protein complexes. 

## INTRODUCTION

Wheat streak mosaic virus (WSMV) has been recognized as an important viral pathogen of wheat. WSMV has filamentous particles of ≈700 nm and is transmitted by the eriophyid mite *Aceria tosichella* Keifer (Brakke, 1987). WSMV has a single-stranded RNA genome of 9384 nucleotides that encodes a single polyprotein (Stenger *et al.,* 1998). The deduced genome organization of WSMV parallels those of other, more studied potyviruses such as tobacco etch virus (TEV; Allison *et al.,* 1986) and tobacco vein mottling virus (TVMV; Domier *et al.,* 1986), but there are, nevertheless, substantial amino acid dissimilarities with these potyviruses. Because of its vector specificity, WSMV was classified as a member of the genus *Rymovirus* in the virus family Potyviridae (Zugula *et al.,* 1992). However, recent phylogenetic studies (Hall *et al.,* 1998; Salm *et al.,* 1996) indicate that the genus *Rymovirus* is not monophyletic, and a reclassification of WSMV into the genus *Tritimovirus* has been proposed (Stenger *et al.,* 1998).

Proteolytic maturation of potyviral polyprotein results in 8–10 proteins (Dougherty and Semler, 1993; Riechmann *et al.,* 1992). A number of studies have assigned one or more functions to the potyviral proteins. P1 is a protein derived from the $NH_2$-terminal region of the polyprotein, and it functions as a proteinase that cleaves at a specific site between itself and helper component-proteinase (HC-Pro) (Verchot *et al.,* 1991). P1 binds to nucleic acids in a sequence-nonspecific manner *in vitro* (Brantley and Hunt, 1993; Soumounou and Laliberte, 1994) and is required for efficient genome amplification (Verchot and Carrington, 1995). Several distinct functions have been defined for HC-Pro of potyviruses. The $NH_2$-terminal region of HC-Pro contains indispensable amino acid residues for the effective transmission of aphid-transmitted potyviruses (Atreya *et al.,* 1992; Atreya and Pirone, 1993; Granier *et al.,* 1993; Huet *et al.,* 1994; Legavre *et al.,* 1996). The COOH-terminal half of HC-Pro functions as a proteinase that cleaves its COOH-terminus in *cis* (Carrington *et al.,* 1989a, 1989b; Oh and Carrington, 1989). In addition, HC-Pro appears to be involved in many other aspects of virus life cycle, such as replication (Kasschau and Carrington, 1995; Kasschau *et al.,* 1997; Klein *et al.,* 1994), long-distance and cell-to-cell movement (Cronin *et al.,* 1995; Kasschau *et al.,* 1997; Rojas *et al.,* 1997), disease synergism (Pruss *et al.,* 1997; Shi *et al.,* 1997), and suppression of post-transcriptional silencing (Anandalakshmi *et al.,* 1998; Kasschau and

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Carrington, 1998). Cylindrical inclusion protein (CI) has RNA helicase and ribonucleotide hydrolysis activities (Fernandez *et al.,* 1995, 1997; Lain *et al.,* 1990, 1991), and the helicase activity is essential for virus RNA replication (Fernandez *et al.,* 1997). In addition, CI is required for both cell-to-cell and long-distance movement (Carrington *et al.,* 1998). Proteolytic cleavage of viral proteins at sites other than the COOH-termini of P1 and HC-Pro is done by a third virus-encoded proteinase, nuclear inclusion protein a (NIa) (Carrington and Dougherty, 1987a, 1987b). NIa or its $NH_2$-terminal half is also the VPg, a viral protein covalently bound to 5′-terminus of genomic RNA (Murphy *et al.,* 1990; Shahabuddin *et al.,* 1988). A 6-kDa protein (6K2) of TEV is membrane associated and is also necessary for virus replication (Restrepo-Hartwig and Carrington, 1994). Nuclear inclusion protein b (NIb) is the most conserved region of the viral polyprotein among the members of *Potyviridae* (Domier *et al.,* 1986; Stenger *et al.,* 1998). Because of the presence of conserved polymerase motifs, NIb is presumed to be the viral RNA-dependent RNA polymerase (Domier *et al.,* 1986, Lain *et al.,* 1989). The coat protein (CP) of potyviruses is encoded in the COOH-terminal region of the polyprotein. Besides its role as a structural protein, CP mediates aphid transmission specificity (Atreya *et al.,* 1991; Atreya *et al.,* 1995; Gal-On *et al.,* 1992) and is required for both cell-to-cell and long-distance movement of potyviruses within infected plants (Dolja *et al.,* 1995; Rojas *et al.,* 1997).

Because multiple potyviral proteins are required for viral replication and spread within the plant host, it is likely that at least some of these functions are mediated by protein–protein interactions. Such interactions also may be important for the regulation of various infection processes as well. Indeed, specific interactions between NIa and NIb of two potyviruses have been demonstrated (Feller *et al.,* 1998; Hong *et al.,* 1995; Li *et al.,* 1997), and the ability of HC-Pro to self-associate has been shown for three potyviruses (Guo *et al.,* 1999; Urcuqui-Inchima *et al.,* 1999). It has also been shown that CP of TVMV binds to HC-Pro *in vitro* (Blanc *et al.,* 1997). Presumably, the proteins encoded by the phylogenetically distinct, mite-transmitted potyviruses function in the same way as the corresponding proteins of the better-characterized aphid-transmitted potyviruses, with the possible exception of viral components required for virus-vector specificity. However, to date there is no direct evidence to support this hypothesis.

To initiate studies on the functions of WSMV-encoded proteins, a preliminary protein interaction map was generated using a yeast two-hybrid system and an *in vitro* protein binding assay. Because the interaction between NIa and NIb has been noted in previous studies (Feller *et al.,* 1998; Hong *et al.,* 1995; Li *et al.,* 1997), this study primarily focused on potential interactions between proteins P1, HC-Pro, P3, and CI. Remarkably, the results presented here indicate that all four proteins not only have the capacity to form homomeric complexes but also have the potential to form almost all possible pairwise heteromeric intermolecular associations, both *in vitro* and *in vivo*.

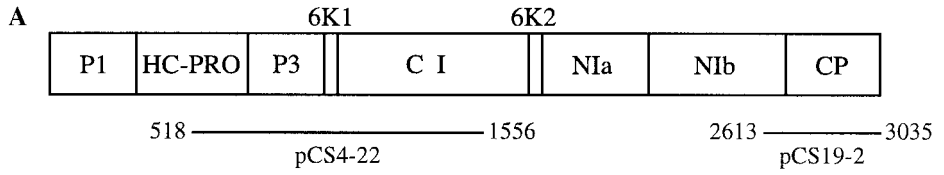
## RESULTS

### Interactions within two groups of proteins encoded in WSMV genome

To gain an initial understanding of the interrelationships among WSMV-encoded proteins, an attempt was made to generate a protein interaction map using a yeast two-hybrid assay. Because the pathway of proteolytic maturation of the WSMV polyprotein has not yet been experimentally determined, we chose a random combinatorial approach. Random libraries of WSMV cDNA were cloned into GAL4 DNA binding domain (BD) and activation domain (AD) vectors. Not unexpectedly, transformation of yeast with the BD library alone resulted in many yeast clones (≈3% of total colonies formed) that could grow on SD/−Trp/−His because they contained self-activating cDNA sequences. To circumvent this problem, such self-activating yeast clones were removed by replica-streaking on SD/−Trp and SD/−Trp/−His.

The BD and AD libraries were simultaneously transformed into yeast cells, and both BD and AD plasmids containing random cDNA sequences were rescued from yeast cells that showed His prototrophy and $\beta$-galactosidase ($\beta$-gal) activity. Several positive yeast clones were chosen for further analysis by DNA sequencing. pCS19-2 and pCS19-11 were BD and AD plasmids, respectively, rescued from one of the positive yeast clones in the two-hybrid assay. pCS19-2 consisted of the cDNA sequence encoding the COOH-terminal region of NIb and the entire CP (Fig. 1A). The cDNA sequence found in pCS19-11 encodes a polyprotein region covering most of NIa, NIb, and CP, excluding the $NH_2$-terminal region of NIa and the COOH-terminal region of CP (Fig. 1B). When the AD library was screened with pCS19-2 as bait, most of the AD plasmids recovered from positive yeast colonies had at least portions of cDNA sequence corresponding to NIb, NIa, or CP, based on restriction nuclease mapping (data not shown). This indicated that most of positive yeast clones in the two-hybrid assay using pCS19-2 arose from the interaction between NIa, NIb, or CP, as previously reported for other members of Potyviridae (Feller *et al.,* 1998; Hong *et al.,* 1995; Li *et al.,* 1997).

Plasmids were also rescued from another positive yeast clone (designated as CS-4) that contained sequences outside of the NIa, NIb, or CP regions. Sequence analyses of cDNA in the plasmids revealed that BD plasmid pCS4-22 contained a cDNA sequence corresponding to a polyprotein region spanning from approximately the middle of HC-Pro to the $NH_2$-terminal three fourths of the predicted CI protein (Fig. 1A). On the other hand, cDNA sequence found in the partner AD




B

| | pCS4-22 | | pCS19-2 | |
|---|---|---|---|---|
| | Growth without His[a] | β-Gal filter assay[b] | Growth without His[a] | β-Gal filter assay[b] |
| pACT2 | – | ND | – | ND |
| pCS4-13 | ++ | + | ++ | + |
| pCS19-11 | – | ND | ++ | + |
| pCS10-1 | ++ | + | +/- | +/- |
| pCS422-4 | + | + | – | ND |
| pCS422-13 | ++ | + | – | ND |
| pCS422-C1 | ++ | + | – | ND |
| pCS192-C1 | – | ND | ++ | + |

FIG. 1. Analysis of multiple pairs of interacting plasmids by the yeast two-hybrid assay. (A) Schematic representation of WSMV polyprotein. The vertical lines denote hypothetical proteolytic cleavage sites organization deduced from the genomic cDNA sequence (Stenger *et al.,* 1998). pCS4-22 and pCS19-2 (shown as lines) were BD plasmids rescued from positive yeast clones obtained in two-hybrid assays of random WSMV cDNA libraries. The amino acid residue coordinates of the WSMV polyprotein encoded by the cDNA clones are indicated at the ends of each line. (B) Two-hybrid assay using multiple pairs of interacting plasmids. pCS4-13 to pCS422-13 were rescued from the AD cDNA library by the interaction with either of pCS4-22 or pCS19-2 in the two-hybrid assay. pCS422-C1 and pCS192-C1 were AD subclones of pCS4-22 and pCS19-2, respectively. Regions of the WSMV polyprotein encoded by respective AD plasmids are indicated by shadowed boxes. Interaction of the AD plasmids with either pCS4-22 or pCS19-2 was evaluated by growth of transformed yeast on SD/−Trp/−Leu/−His ([a]scored as ++, colonies ≥1 mm in diameter formed; +, colonies ≥1 mm in diameter formed, but the number of colonies on SD/−Trp/−Leu/−His was <30% of that on SD/−Trp/−Leu; ±, inconsistent appearance of several colonies of ≥1 mm; −, no colonies of ≥1 mm in diameter formed) and [b]β-gal colony lift filter assay (scored as +, blue color developed within 2 h; ±, variable positive results; ND, not determined).

plasmid, pCS4-13, encoded a protein consisting of the COOH-terminal region of CI and most of NIa, excluding the predicted COOH-terminus (Fig. 1B). To better define the protein domains involved in the interaction, the AD library was screened again with pCS4-22. This two-hybrid assay, however, resulted in only a few positive yeast colonies. Among them, three interacting AD plasmids were isolated and characterized by DNA sequencing

(Fig. 1B). pCS10-1 has a cDNA sequence corresponding to the COOH half of 6K1 and approximately one third of CI from the $NH_2$-terminus. The cDNA fragment in pCS422-4 encoded a polyprotein region covering approximately three fourths of HC-Pro from the COOH-terminus to one third of CI from the $NH_2$-terminus. However, yeast containing pCS422-4 with pCS4-22 had significantly reduced His prototrophy compared with yeast transformed with pCS4-22 and pCS10-1. The protein encoded by the cDNA in pCS422-13 contained the COOH-terminal region of CI.

To determine whether the interactions of the four AD plasmids (pCS4-13, pCS10-1, pCS422-4, and pCS422-13) were specific for pCS4-22, each AD plasmid was also tested with pCS19-2 in yeast cells. In this two-hybrid assay, only pCS4-13, which encodes a protein extending from the COOH-terminal region of CI through most of NIa, strongly interacted with pCS19-2 (Fig. 1B). After analysis of these multiple pairs of interacting clones, it became apparent that the two-hybrid interactions identified fell into two distinct groups of proteins encoded by WSMV. The first group of interacting proteins contained primarily NIa, NIb, and CP, and the second type of interactions involved HC-Pro, P3, or CI. A representative clone of this latter group, pCS4-22, contained about half of HC-Pro and extended through P3 into much of CI. Because the COOH half of HC-Pro in TEV retains autoproteolytic activity in *Escherichia coli* and *in vitro* (Carrington *et al.,* 1989a, 1989b), the fusion protein produced in yeast harboring pCS4-22 could consist of the GAL4 DNA binding domain followed by the COOH half of HC-Pro if a similar self-processing event were to occur. Thus the second type of interaction found in CS-4 might involve only HC-Pro and CI. Similarly, autoproteolysis by NIa (Carrington and Dougherty, 1987a, 1987b) may occur for the fusion proteins encoded in pCS4-13 and pCS19-11, leaving only portions of CI or NIa, respectively, in the resulting AD fusion proteins. These possibilities were explored by immunoblotting and deletion analyses (see below).

## Proteolytic cleavage of WSMV polyproteins in yeast cells

Due to the presence of multiple protein domains including likely proteinase regions in clones initially recovered by the two-hybrid assay (Fig. 1), identification of protein domains interacting in yeast cells required further experimentation. To determine whether proteolytic cleavage actually occurs, we first examined the size of the fusion proteins containing proteinase domains produced *in vivo* by immunoblot analyses. Other cDNA clones containing fusion proteins that terminate at the predicted cleavage sites were constructed and included in these experiments as controls. As shown in Figs. 2B and 2D, the fusion proteins encoded in pCS19-11 and pCS4-22 appeared to undergo autoproteolysis in yeast. The size of the AD protein derived from pCS19-11 (Fig. 2B, lane 4) was the same as that from pCS1911-NIa (Fig. 2B, lane 3), which encodes only the NIa portion of pCS19-11. Likewise, the BD protein detected from yeast cells harboring pCS4-22 (Fig. 2D, lane 4) was the same size as that produced from pCS422-S5 (Fig. 2D, lane 3), which has only cDNA for the HC-Pro portion of pCS4-22. On the other hand, yeast cells transformed with pCS4-13 accumulated different AD fusion proteins (Fig. 2C, lane 4). The smaller protein of the two was the same size as the protein encoded in pCS413-CIC (Fig. 2C, lane 3), which consists of GAL4 AD and the CI portion of pCS4-13. The other form of fusion protein has a molecular mass (≈80 kDa) consistent with that of an unprocessed translation product of pCS4-13. The protein produced from pCS19-2 contains the site of proteolytic cleavage (NIb/CP) by NIa in *trans*. pCS19-2 was able to interact with both pCS4-13 and pCS19-11 (Fig. 1B). Proteolytic cleavages of the protein from pCS19-2 in the presence of pCS4-13 or pCS19-11 were also examined by immunoblot analysis. The size of BD protein from yeast transformed with pCS19-2 and pCS4-13 (Fig. 2E, lane 6) was the same as that from pCS192-NIb consisting of only the NIb portion of pCS19-2 and GAL4 BD (Fig. 2E, lane 3). Thus the fusion protein of pCS19-2 was apparently cleaved in the presence of pCS4-13, although noncleaved product was also detected. In contrast, the BD protein of pCS19-2 was only partially cleaved by the AD protein encoded by pCS19-11 (Fig. 2E, lane 5). The BD protein of pCS19-2 appeared as a spectrum of cleavage products in the presence of pCS19-11.

These results provide the first experimental evidence that WSMV HC-Pro and NIa are proteinases and partially map the respective protein domains required for proteolytic activity. Thus the COOH-terminal region (518–736) of HC-Pro retains proteolytic activity for *cis*-cleavage. Similarly, amino acid residues 1762–2167 (CS4-13) or 1955–2187 (CS19-11) of NIa each have the ability to cleave in *cis*. The results also imply that only CS4-13 encompasses those protein regions required to function properly for *trans* cleavage, however.

These results also suggest that the interacting domains of proteins expressed from pCS4-22 and pCS19-11 were the COOH-terminal half of HC-Pro and the NIa portion of pCS19-11, respectively. However, it was still unclear which domains of the protein produced from pCS4-13 were interacting with those from pCS4-22 or pCS19-11, because both cleaved and apparently noncleaved forms of fusion proteins encoded by pCS4-13 are also present *in vivo* (Fig. 2C).

## Subdomains of CI, HC-Pro, and P1 interact with one another in yeast cells

Based on the results obtained by the initial two-hybrid assay (Fig. 1) and immunoblot analyses (Fig. 2), we

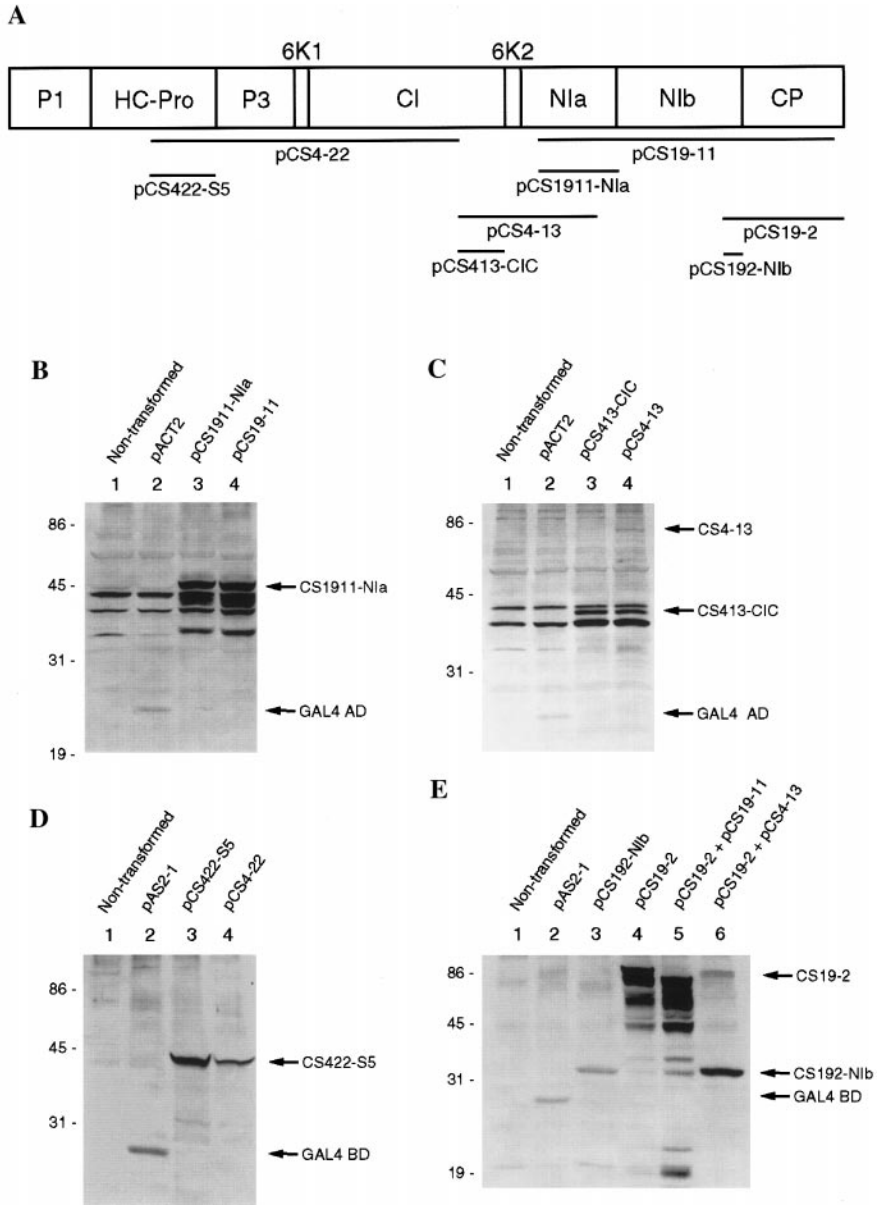
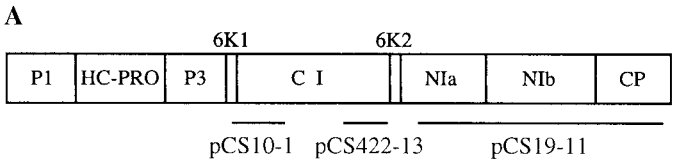



FIG. 2. Proteolytic cleavage of GAL4 AD and BD fusion proteins by WSMV proteinase domains in yeast. Total protein samples were obtained from yeast transformed with GAL4 AD or BD plasmids depicted in panel A and subjected to immunoblot analyses. (A) WSMV cDNA coding regions of AD or BD plasmids used for transformation of yeast are indicated with lines under the schematic representation of WSMV polyprotein. (B–D) *cis*-Proteolytic cleavage of fusion proteins expressed from pCS19-11, pCS4-13, and pCS4-22. Protein samples prepared from yeast cells transformed with plasmids indicated on the top of each panel were probed with anti-GAL4 AD (B and C) or anti-BD (D) antibodies. Positions of proteins produced from respective plasmids are indicated with arrows to the right of each panel. The size (kDa) and positions of a protein molecular weight standard are shown on the left. (E) Proteolytic cleavage of the fusion protein from pCS19-2 in *trans*. Yeast cells were transformed with plasmids or combinations of plasmids as indicated on the top of the panel. Protein samples from the transformed yeast cells were probed with anti-BD antibodies.

further analyzed interactions of deletion derivatives of pCS4-22 and pCS4-13 in yeast to confirm the specific interaction between subdomains of HC-Pro and CI. As expected, the protein from pCS422-S5 (HC-Pro portion of pCS4-22) was able to interact with those of pCS422-13 (COOH-terminal region of CI) and pCS10-1 (6K1/CI $NH_2$-terminal region) (Fig. 3B). The cDNA fragment of pCS4-22 was then split into two fragments at a *Nco*I site near the predicted COOH-terminal of HC-Pro, and each cDNA fragment was subcloned into pAS2-1 to obtain pCS422-S1 and pCS422-S3 (Fig. 3B). The cDNA fragment in pCS422-S1 encodes almost all of the COOH-terminal half of HC-Pro, except the last 11 amino acid residues, whereas pCS422-S3 has the cDNA fragment for a polyprotein region from the last 12 amino acid residues of HC-Pro to approximately the $NH_2$-terminal three fourths of CI. When pCS422-S1 and -S3 were tested by the two-hybrid assay in the presence of pCS10-1 or

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| | pCS422-13 | | pCS10-1 | | pCS19-11 | |
|---|---|---|---|---|---|---|
| | Growth without His[a] | β-Gal filter assay[b] | Growth without His[a] | β-Gal filter assay[b] | Growth without His[a] | β-Gal filter assay[b] |
| pCS4-22 | ++ | + | ++ | + | – | ND |
| pCS422-S1 | – | ND | – | ND | – | ND |
| pCS422-S2 | ++ | + | ++ | + | – | ND |
| pCS422-S3 | – | ND | – | ND | – | ND |
| pCS422-S5 | ++ | + | ++ | + | – | ND |
| pSHCPRO-C | – | ND | + | + | – | ND |
| pSHCPRO-F | ++ | + | ++ | + | – | ND |

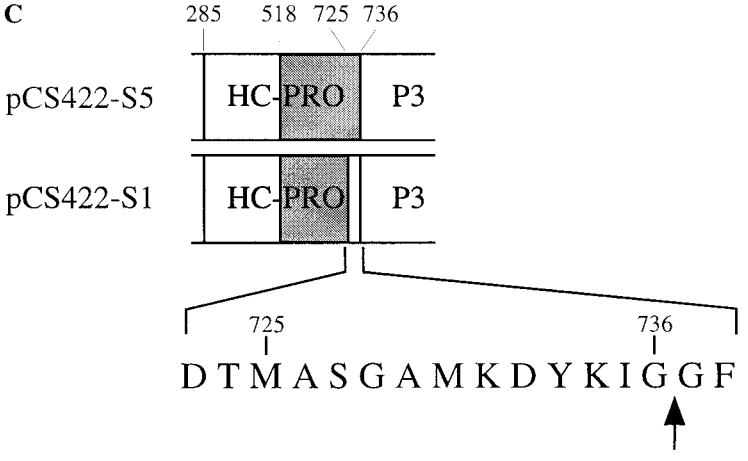


FIG. 3. Analysis of deletion clones derived from pCS4-22 by the two-hybrid assay. (A) WSMV protein regions encoded in AD plasmids used in the yeast two-hybrid assay. Underlines indicate the encoded regions of each plasmid. (B) Schematic diagrams of protein regions encoded by pCS4-22, deletion clones of pCS4-22 (pCS422-S1 to pSHCPRO-C), and pSHCPRO-F are depicted on the left. WSMV encoded regions contained in the clones are indicated by shadowed boxes. Interactions of the BD plasmids (pCS4-22 to pSHCPRO-F) with AD plasmids (pCS422-13, pCS10-1, and pCS19-11)

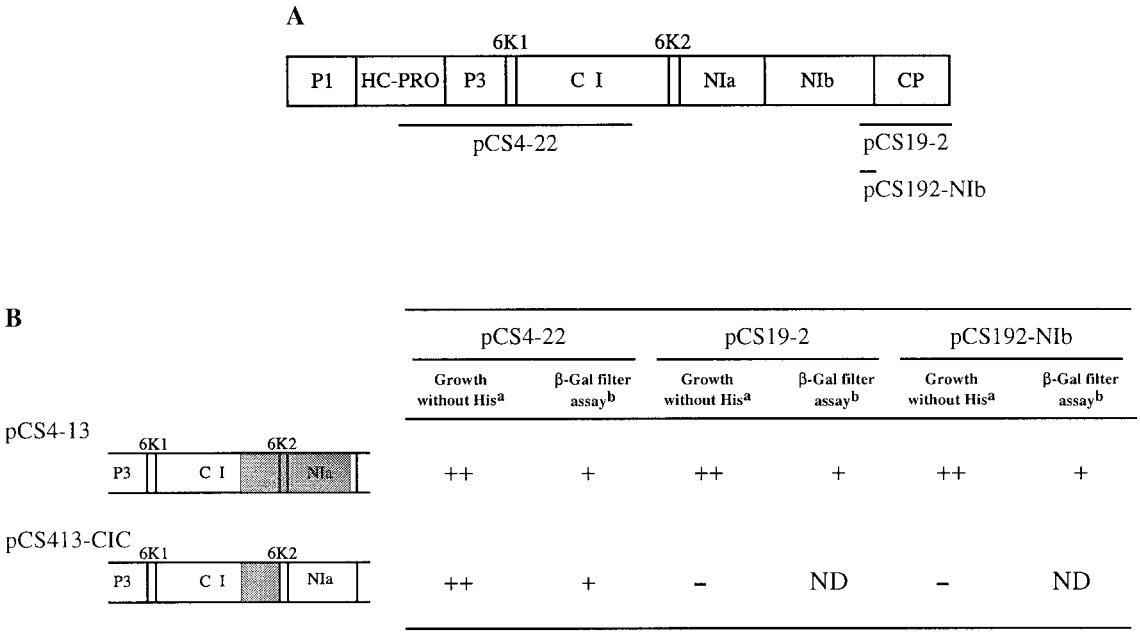


FIG. 4. Deletion analysis of pCS4-13 by the two-hybrid assay. (A) WSMV protein regions encoded in BD plasmids pCS4-22, pCS19-2, and pCS192-NIb were indicated as lines under the schematic diagram of the WSMV polyprotein. (B) The cDNA coding region of pCS4-13 and pCS413-CIC are depicted on the left with shadowed boxes. The reactions with pCS4-22, pCS19-2 ,and pCS192-NIb by the two-hybrid assay are shown on the right. Interactions were evaluated by [a]the growth of transformed yeast on SD/−Trp/−Leu/−His and [b]$\beta$-gal colony-lift assay (scored as in Fig. 1).

pCS422-13, none of the plasmid combinations produced yeast colonies on SD/−Trp/−Leu/−His (Fig. 3B). On the other hand, pCS422-S2 (which encodes a fusion protein predicted to self-cleave to a product the same size as that of pCS422-S5) was found to interact with both pCS10-1 and pCS422-13 in the yeast two-hybrid assay (Fig. 3B).

Together, these results suggested that the difference in the HC-Pro portions of pCS422-S1 and -S5 (i.e., 11 amino acid residues at the HC-Pro COOH-terminus), was important in the interaction (Fig. 3C). To test this hypothesis, an additional plasmid construct, pSHCPRO-C, was made. The cDNA sequence in pSHCPRO-C encodes 79 amino acid residues from the COOH-terminus of HC-Pro. pSHCPRO-C interacted weakly with pCS10-1 but not at all with pCS422-13 (Fig. 3B). Therefore, these results are compatible with the hypothesis that the interacting region of pCS4-22 consists of the COOH-terminal half of HC-Pro, and the 11 amino acid residues in the COOH-terminus of HC-Pro are indispensable, but not sufficient, for the interaction with the COOH-terminal, and possibly the $NH_2$-terminal, regions of CI (see later). These results were also consistent with the results from immunoblot analysis (Fig. 2D), suggesting that the BD protein produced from pCS4-22 accumulate in yeast as a processed form (GAL4 BD/COOH half of HC-Pro).

As shown in Fig. 2C, it was likely that a fraction of the protein expressed from pCS4-13 exists as unprocessed form (partial CI/6K2/partial NIa) in yeast cells. Therefore, to define interacting regions of the protein produced from pCS4-13, pCS413-CIC, which has only cDNA sequence for the CI portion of pCS4-13, was tested for the interaction in yeast in the presence of pCS4-22 or pCS19-2. The results are summarized in Fig. 4, showing that pCS413-CIC was able to interact with pCS4-22 but not with pCS19-2 or pCS192-NIb. This result indicates that the region of protein encoded in pCS4-13 interacting with that of pCS4-22 is the COOH-terminal region of CI, whereas the BD proteins produced from pCS19-2 or pCS192-NIb might interact with the NIa and/or 6K2 portions encoded in pCS4-13. The fact that two independent clones pCS413-CIC and pCS422-13 (Fig. 1B) are able to interact with pCS4-22 (or pCS422-S5) clearly demonstrates that the COOH-terminal region of CI specifically interacts with the COOH-terminal half of HC-Pro.

To further elucidate potential protein interactions, a

shown on the right were evaluated by [a]the growth of transformed yeast on SD/−Trp/−Leu/−His and [b]$\beta$-gal colony lift filter assay (scored as in Fig. 1). (C) Diagram detailing the difference between the COOH-termini of pCS422-S5 and pCS422-S1. Protein regions encoded by pCS422-S5 and pCS422-S1 are also indicated by shadowed boxes. Amino acid residue number of the predicted $NH_2$-terminus of HC-Pro (285), the $NH_2$-termini of the protein region in pCS422-S5 and -S1 (518), the COOH-terminus of the protein region in pCS422-S1 (725) and the COOH-termini of the protein region in pCS422-S5 and HC-Pro (736) are indicated above the diagram. Amino acid residues 723–738 are shown as one-letter code. The arrow indicates the predicted *cis*-cleavage site for HC-Pro.

TABLE 1

Interactions of Truncated WSMV Proteins Detected by the Yeast Two-Hybrid Assay[a]

| BD fusion plasmid (coding region) | AD fusion plasmid (coding region) |
|---|---|
| pCS422-S5 (HC-Pro COOH-terminal half) | pCCI-N (CI $NH_2$-terminal region) |
| | pCP1-F (full-length P1) |
| | pCS422-13 (CI COOH-terminal region) |
| | pCS422-C5 (HC-Pro COOH-terminal half) |
| pSCI-F (full-length CI) | pCS422-13 (CI COOH-terminal region) |
| | pCS10-1 (6K1/CI $NH_2$-terminal region) |
| pSP1-F (full-length P1) | pCS422-13 (CI COOH-terminal region) |
| | pCS10-1 (6K1/CI $NH_2$-terminal region) |

[a] Yeast cells transformed with a pair of plamids (left and right columns) grew on SD/-Trp/-Leu/-His and developed blue color in $\beta$-gal colony-lift assay. Only positive interactions are listed.

second approach was taken. Rather than using random cDNA libraries, the two-hybrid assay was performed using defined, full-length cDNAs of CI, HC-Pro, and P3, which were generated by PCR according to the predicted cleavage sites (Stenger *et al.,* 1998). Although no clones containing cDNA sequences of WSMV P1 were isolated in the initial screens, full-length P1 cDNA was also included in this two-hybrid assay. Based on the previous results, the CP gene was also tested as a negative control. Surprisingly, except for weak homologous interactions identified between CI, P1, P3, and CP, no heterologous interactions were detected between any of the full-length proteins by the two-hybrid assay (data not shown).

Despite the lack of clear evidence of heterologous interactions between the full-length proteins in both AD and BD vectors, full-length proteins of CI and P1 produced from the BD vector were able to interact with the COOH-terminal region of CI (pCS422-13) in yeast cells (Table 1). Also, interactions were apparent between pCS10-1 (partial 6K1/NH2-terminal region of CI) and either of pSCI-F (full-length CI) and pSP1-F (full-length P1), suggesting portions of 6K1 and/or the $NH_2$-terminal region of CI were involved in the interaction with CI and P1 (Table 1). In addition, pCS422-S5 interacted with AD plasmids encoding either full-length P1 (pCP1-F), the $NH_2$-terminal region of CI (pCCI-N), and the COOH half of HC-Pro (pCS422-C5). In contrast, AD plasmids containing full-length sequences of CP (pCCP-F) or P3 (pCP3-F) did not give rise to colonies on SD/−Trp/−Leu/−His when introduced into yeast with either pCS10-1 or pCS422-13.

Based on the results obtained by the two-hybrid assay using truncated and full-length proteins of WSMV, multiple interactions were clearly identified among subdomains of CI, HC-Pro, and P1 in yeast. The COOH half of HC-Pro was shown to have the capacity to participate in interactions with itself, with both $NH_2$- and COOH-terminal regions of CI, and with P1. The $NH_2$- and COOH-terminal regions of CI were each found to have the capacity to bind to full-length forms of P1 and CI (Table 1).

### CI, HC-Pro, P1, and P3 bind with each other *in vitro*

The previous results made it likely that both termini of CI interacted independently with HC-Pro and P1, but the two-hybrid assay failed to show interactions between the respective heterologous full-length proteins. To obtain independent evidence that specific interactions could in fact occur among these proteins, an alternative experimental approach was explored. *In vitro* protein binding assays were performed using glutathione-*S*-transferase (GST) fusion proteins and *in vitro* translation products. Full-length CI, HC-Pro, P1, P3, and CP were expressed as GST fusion proteins in *E. coli* (Fig. 5) and immobilized on glutathione–Sepharose. *In vitro* translated proteins were then added to observe potential protein binding. Full-length CI bound to both HC-Pro and P1 *in vitro* (Fig. 6, A5, A7, B4, and D4). In addition to binding with CI, P1 and HC-Pro also had the capacity to bind to each other (Fig. 6, B7 and D5). P3, which gave no apparent interactions with any regions of the polyprotein tested other than with itself in the two-hybrid assay, bound to CI, HC-Pro, and P1 as well as itself in the *in vitro* assay (Fig. 6, A8, B8, D8, E4, E5, E7, and E8). The *in vitro* translations of all WSMV-encoded cistrons contained truncated products, likely due to initiation of translation at internal, in-frame AUG codons, which retained the binding properties of their full-length counterparts. Binding of these may be due to direct interaction with the GST fusion proteins or may be due to indirect associations mediated by homologous interaction of truncated and full-length proteins followed by binding of the latter to the GST fusion proteins. Compared with the binding activities among various pairwise combinations of CI, HC-Pro, P1, and P3 described above,

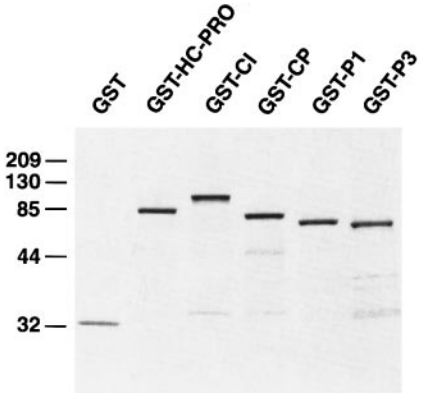


FIG. 5. SDS–PAGE of partially purified WSMV-encoded proteins expressed as fusions with GST in bacteria. Protein extracts from bacterial suspensions were affinity purified on glutathione–Sepharose columns. The positions of Bio-Rad protein molecular mass standards after electrophoresis and their molecular masses are shown on the left side of the figure. The gel was stained with Coomassie blue.

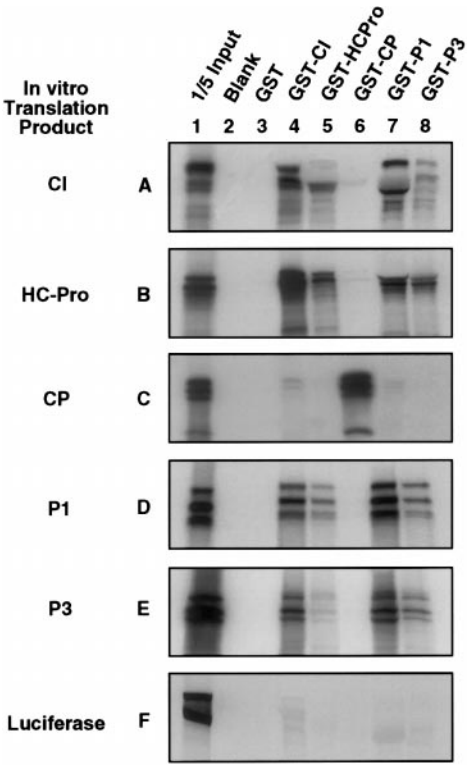


FIG. 6. *In vitro* binding assay of WSMV-encoded proteins. GST (lane 3) or fusion proteins of GST and WSMV-encoded proteins (lanes 4–8) immobilized on glutathione–Sepharose were mixed with *in vitro* translation products from WSMV genes (A–E) or a luciferase gene provided as an *in vitro* translation control (F). After incubation, samples were analyzed by 12% SDS–PAGE followed by autoradiography. One fifth of the *in vitro* translation products used for the binding assay was loaded in lane 1 of each panel. Translation products appeared as a spectrum of full-length, internally initiated, and prematurely terminated proteins. Note that distortion of autoradiography bands (A5, A7, A8, B7, and B8) was due to overlapping migration of excess unlabeled GST fusion proteins on the gels.

their interaction with CP was negligible (Fig. 6, A6, B6, C4, C5, C7, C8, D6, and E6). Binding between the GST fusion proteins and *in vitro* translated luciferase was also negligible (Fig. 6, F), and none of the viral *in vitro* translation products bound to immobilized GST (Fig. 6, lanes 3 of A–F). In addition, each protein tested was able to form homomeric complexes *in vitro* (Fig. 6, A4, B5, C6, D7, and E8).

Taking all the results together, it is evident that full-length CI, HC-Pro, P1, and P3 contain domains that can participate in a complex network of binding interactions with each other, although the levels of interaction may differ among specific protein pairs. Importantly, the *in vitro* binding results both substantiated and extended the findings of the two-hybrid assay, thus confirming the heterologous interactions between truncated CI and both HC-Pro and P1, as well as the homologous interactions of CI, CP P1, and P3.

The interactions between WSMV-encoded proteins observed in this study are summarized in Fig. 7. Heterologous interactions among P1, CI, and HC-Pro and homologous interactions of P1, P3, HC-Pro, CI, and CP were detected by both the two-hybrid and the *in vitro* binding assays, whereas the interactions of P3 with other proteins were identified only by the *in vitro* protein binding assay.

## DISCUSSION

A remarkably complex potential for homologous and heterologous associations among five WSMV proteins was revealed using two independent assay systems for detecting protein–protein interactions. WSMV P1, HC-Pro, P3, CI, and CP all have the capacity to self-associate, and all except CP were shown to be able bind each other in all possible pairwise combinations. Importantly, a number of these interactions are consistent with the known properties of proteins encoded by other potyviruses. The ability of HC-Pro to self-associate has been shown for potato virus A (PVA), potato virus Y (PVY), and lettuce mosaic virus (Guo *et al.,* 1999; Urcuqui-Inchima *et al.,* 1999). P1 of PVY and P3 of TVMV were found in association with cylindrical inclusion bodies (Arbatova *et al.,* 1998; Rodriguez-Cerezo *et al.,* 1993), whereas P3 of TEV was found in nuclear inclusions (Langenberg and Zhang, 1997). P1 and P3 of TVMV colocalize to the same membrane-rich fractions from infected leaf extracts (Rodriguez-Cerezo and Shaw, 1991). Both CI and HC-Pro of potyviruses are present near plasmodesmata (Langenberg, 1986; Roberts *et al.,* 1998; Rodriguez-Cerezo *et al.,* 1997; Rojas *et al.,* 1997). These observations might reflect the physical association of these proteins in plant cells. Furthermore, the potyviral proteins CI, HC-Pro, CP, and P1 have been demonstrated to play multiple, interrelated roles during infection. As noted previously, CI and HC-Pro, along with CP, have been proposed to be involved in several aspects of virus movement within the plant (Carrington *et al.,* 1998; Cronin *et al.,* 1995; Dolja *et al.,* 1995;

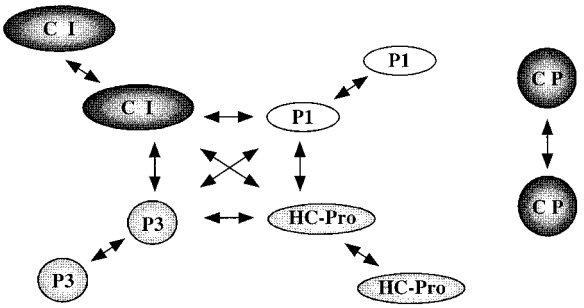


FIG. 7. Interactions among P1, HC-Pro, P3, CI, and CP encoded by WSMV genome. Interactions are indicated by arrows between schematic representation of the WSMV-encoded proteins. The interactions were identified by the two-hybrid and the *in vitro* protein binding assays, except heterologous interactions of P3 with P1, HC-Pro, and CI, which were detected by only the *in vitro* binding assay.

Kasschau *et al.,* 1997; Langenberg, 1986; Roberts *et al.,* 1998; Rodriguez-Cerezo *et al.,* 1997; Rojas *et al.,* 1997), whereas mutations in CI (Carrington *et al.,* 1998; Fernandez *et al.,* 1997; Klein *et al.,* 1994), HC-Pro (Kasschau and Carrington, 1995; Kasschau *et al.,* 1997; Klein *et al.,* 1994), P1 (Klein *et al.,* 1994; Verchot and Carrington, 1995), and P3 (Klein *et al.,* 1994) have been shown to abolish or greatly reduce the extent of viral RNA replication. Taken together, the manifold spatial and functional associations among these proteins are consistent with the complex pattern of WSMV protein–protein interactions identified here. More recently, a similar complex of interactions among potyviral proteins was identified for PVA using yeast two-hybrid, overlay blotting, and liquid binding assays (Mertis *et al.,* 1999).

Some aspects of protein function gained by the study of aphid-transmitted potyviruses, however, may not always be applicable for the corresponding WSMV encoded proteins. For example, there was no apparent interaction between CP and HC-Pro of WSMV (Fig. 6), whereas an interaction between CP and HC-Pro has been observed with TVMV (Blanc *et al.,* 1997). Furthermore, the amino-terminal region of HC-Pro in aphid-transmitted potyviruses is important for transmission (Atreya *et al.,* 1992; Atreya and Pirone, 1993; Blanc *et al.,* 1997; Granier *et al.,* 1993; Legavre *et al.,* 1996) and encompasses a major domain for self-interaction for HC-Pro of PVA (Guo *et al.,* 1999), lettuce mosaic virus, and PVY (Urcuqui-Inchima *et al.,* 1999). In contrast, a major domain for self-interaction of WSMV HC-Pro resides in the COOH half (Table 1). This suggests that at least some functions of HC-Pro may differ among aphid- and mite-transmitted potyviruses, particularly because the amino-terminal half of HC-Pro of WSMV is more divergent from HC-Pro of other potyviruses than is the COOH half (Stenger *et al.,* 1998).

The yeast two-hybrid assay system (Fields and Song, 1989) has emerged as a powerful tool to identify protein–protein interactions, and it already has been used to catalog the interactions among a variety of viral gene products (Bartel *et al.,* 1996; Cuconati *et al.,* 1998; Hong *et al.,* 1995). Despite a number of advantages, the two-hybrid assay also has limitations (Bai and Elledge, 1997). The failure of the two-hybrid assay using with full-length WSMV gene products is similar to the inconsistency observed for interactions among poliovirus nonstructural proteins (Cuconati *et al.,* 1998). Protein 2C of poliovirus, which may be a functional homolog of the potyviral CI (Rodriguez and Carrasco, 1993, 1995), failed to interact with itself in the two-hybrid assay, yet it formed homologous multimers *in vitro*. Furthermore it was also shown the relative levels of poliovirus 2B/2C interaction detected by the two assay systems were not correlated (Cuconati *et al.,* 1998). Given that WSMV CI protein is known to form cylindrical inclusions within plant cells, the lack of a homologous interaction of full-length CI protein in the two-hybrid assay provides a clear example of the limitations of the two hybrid system as applied to potyviruses.

The use of random WSMV cDNA libraries in two hybrid assays allowed a relatively unbiased search for interacting protein domains. As with other viruses that are translated as polyproteins and undergo subsequent proteolytic processing, the complexity of the protein fusion library is reduced whenever the cloned cDNAs encompass both a proteinase and a cleavage site. All three potyviral proteinases, P1, HC-Pro, and NIa, are capable of autoproteolysis (Carrington and Dougherty, 1987a, 1987b; Carrington *et al.,* 1989a, 1989b; Dougherty and Semler, 1993; Verchot *et al.,* 1991). Therefore it is quite possible that GAL4 fusions spanning these proteinase domains may be self-processed in yeast as well. In this study, we did find compelling evidence that HC-Pro and NIa of WSMV are indeed proteinases. For example, the predicted polyprotein encoded by pCS4-22 has the HC-Pro proteinase domain that processes in *cis*, resulting in a BD fusion protein with an exact HC-Pro COOH-terminus (Fig. 2). Consistent with this observation, the patterns of interaction of pCS4-22 and pCS422-S5 with pCS422-13 and pCS10-1 (Fig. 3) were the same, and deletion analysis of pCS4-22 revealed that the interacting region mapped exactly to the COOH half of HC-Pro. In the same way, the interaction between pCS4-22 and pCS422-4 might be mediated by direct interaction of processed HC-Pro termini, because pCS422-4 encodes an even larger portion of HC-Pro than that of pCS4-22. Clones containing an NIa proteinase domain, such as pCS4-13, have both *cis* and *trans* proteinase activities, whereas the proteinase domain in pCS19-11 was active only in *cis*, as revealed by immunoblot analyses of fusion proteins in yeast (Fig. 2).

The COOH-terminal region of CI (pCS422-13) interacted with CI, HC-Pro, and P1 in yeast (Table 1). These mutual protein associations were corroborated by the *in vitro* binding assays with full-length proteins. Finer mapping within the COOH-terminal domain of CI will reveal whether interacting regions for the respective proteins overlap or are independent. Also, it would be of particular interest to see whether any of the interactions among these WSMV proteins are mutually exclusive or noncompetitive *in vitro*.

The interactions detected in this study must be reexamined in plant cells, perhaps through the use of engineered virus or plant genomes, before functional relationships can be definitively assigned to these proteins. Nevertheless, the capacity to form homomeric and heteromeric molecular interactions is consistent with the pleiotropic nature of many potyviral gene mutants, suggests potential mechanisms for the regulation of various viral processes via a network of viral protein complexes, and provides potential new targets for the inhibition of potyviral replication.

## MATERIALS AND METHODS

### Production of WSMV cDNA

Virions of WSMV (isolate Sidney 81) were partially purified by the method of Lane (1986). Viral RNA was recovered by treatment of virions with proteinase K followed by phenol–chloroform extraction and ethanol precipitation. Polyadenylated viral RNA was further purified using the PolyA Tract I mRNA isolation kit (Promega, Madison, WI). cDNA was synthesized from the viral RNA with oligo(dT) or random hexamer primers using a cDNA synthesis kit (Pharmacia Biotech, Piscataway, NJ).

### Yeast two-hybrid assay

All media, buffers, and methods for the yeast two-hybrid assay were adopted from the MATCHMAKER System 2 protocol (Clontech, Palo Alto, CA). Random libraries of WSMV cDNA were made in both the GAL4 DNA binding domain (pAS2-1, BD library) and the activation domain (pACT2, AD library) vectors. Spurious, self-activating cDNA clones in the BD library were eliminated by replica-streaking. Approximately 6000 independent yeast (*Saccharomyces cerevisiae* strain CG-1945) colonies transformed with the BD library were streaked onto a synthetic medium lacking tryptophan (SD/−Trp) and incubated for 3–5 days. The streaked yeast cells were then transferred onto a synthetic medium lacking tryptophan and histidine (SD/−Trp/−His). Yeast cells that could grow on SD/−Trp but could not grow on SD/−Trp/−His were pooled and used as the source of the BD library. Yeast cells were simultaneously transformed with the BD and the AD libraries and evaluated for prototrophy for histidine (His) by growing on the synthetic medium lacking tryptophan, leucine, and histidine (SD/−Trp/−Leu/−His). Cells that grew on SD/−Trp/−Leu/−His were then tested for the expression of $\beta$-gal by colony-lift filter assay (Bai and Elledge, 1997). Plasmids were rescued from yeast colonies that were positive for both His prototrophy and $\beta$-gal expression. Rescued plasmids were initially characterized by restriction nuclease mapping, followed by DNA sequence analysis.

### Plasmid construction

The plasmids used in this study are given in Table 2. The regions of WSMV proteins encoded in each plasmid are identified by beginning and ending amino acid coordinates of the WSMV polyprotein.

*Plasmids rescued from transformed yeast.* pCS4-22 and pCS19-2 were WSMV cDNAs in the BD vector (pAS2-1), whereas pCS4-13, pCS422-4, pCS422-13, pCS10-1, and pCS19-11 were cDNAs in the AD vector (pACT2), all of which were rescued from positive yeast clones resulting from a two-hybrid assay. These were found to contain the following WSMV sequences (GenBank accession number AF057533): pCS4-22, nucleotide (nt) positions 1682–4799; pCS19-2, 7967–9390; pCS4-13, 4797–6633; pCS422-4, 1323–4034; pCS422-13, 4710–5259; pCS10-1, 3231–3959; and pCS19-11, 5993–8980.

TABLE 2

Amino Acid Coordinates of Predicted WSMV Proteins and Proteins Encoded in Plasmids Used in the Study

| WSMV proteins | |
|---|---|
| P1 | 1–284 |
| HC-Pro | 285–736 |
| P3 | 737–1014 |
| 6K1 | 1015–1066 |
| CI | 1067–1710 |
| 6K2 | 1711–1761 |
| NIa | 1762–2187 |
| NIb | 2188–2686 |
| CP | 2687–3035 |
| **Binding domain plasmids** | |
| pSP1-F | 1–284 |
| pSHCPRO-F | 285–736 |
| pSP3-F | 737–1014 |
| pSCI-F | 1067–1710 |
| pSCP-F | 2687–3035 |
| pCS4-22 | 518–1556 |
| pCS422-S1 | 518–725 |
| pCS422-S2 | 518–1000 |
| pCS422-S3 | 726–1556 |
| pCS422-S5 | 518–736 |
| pSHCPRO-C | 658–736 |
| pCS19-2 | 2613–3035 |
| pCS192-NIb | 2613–2686 |
| **Activation domain plasmids** | |
| pCP1-F | 1–284 |
| pCHCPROF | 285–736 |
| pCP3-F | 737–1014 |
| pCCI-F | 1067–1710 |
| pCCP-F | 2687–3035 |
| pCS4-13 | 1557–2167 |
| pCS422-4 | 399–1301 |
| pCS422-13 | 1528–1710 |
| pCS10-1 | 1035–1276 |
| pCS19-11 | 1955–2950 |
| pCS422-C1 | 518–1556 |
| pCS192-C1 | 2613–3035 |
| pCS422-C5 | 518–736 |
| pCS413-CIC | 1557–1710 |
| pCS1911-NIa | 1955–2187 |
| **Plasmids for GST fusion protein** | |
| pGEX-P1 | 1–284 |
| pGEX-HCPRO | 285–736 |
| pGEX-P3 | 737–1014 |
| pGEX-GI | 1067–1710 |
| pGEX-CP | 2687–3035 |

*Plasmids for full-length protein interactions.* cDNA fragments for full-length CI (nt position 3329–5259), HC-Pro (983–2338), CP (8189–9390), P1 (131–982), and P3 (2339–3172) were amplified by polymerase chain reaction (PCR) with *Pfu* polymerase (Stratagene, La Jolla, CA) from the cDNA library or plasmids containing appropriate cDNA sequences using the following oligonucleotides: CI, 5′-GTGGATCCCCGGA ATTTTTGAAGATCTG-3′ (CI-5) and 5′-GTGGATCCTACTGGTATGACACA TGGG-3′ (CI-3); HC- Pro, 5′-CCGAATCCCCAGTGGCATACAATAC-3′ (HCPRO-5) and 5′-CCGGATCCTCAGCCAATTTTGTAATCTTT-3′ (HCPRO-3); CP, 5′-CCGGATCCCCTCGAGTGAGGCCGCAACC-3′ (CP-5) and 5′-GGGG ATCCTTCGCCCGGAATTAGCTTG-3′ (CP-3); P1, 5′-CCGGATCCAATGGC AACAGCGAATTGT-3 (P1–5), and 5′-CCGGATCCCTAATAAGTTGTGAT AAAGCC-3′ (P1–3); and P3, 5′-GCGGATCCGCGGGTTCCAAGAGACTGTT-3′ (P3–5) and 5′-CCGGATCCTATTGGTATTCAACCAATTC-3′ (P3–3). The amplified DNA fragments for CI, CP, P1, and P3 were digested with *Bam*HI and inserted into pAS2-1 to produce pSCI-F, pSCP-F, pSP1-F, and pSP3-F, respectively. Likewise, pCCI-F, pCCP-F, pCP1-F, and pCP3-F were constructed by ligating pACT2 and the full-length, *Bam*HI fragments of CI, CP, P1, or P3. The DNA fragment for HC-Pro was digested with *Bam*HI and inserted into *Sal*I-filled-in, *Bam*HI-digested pAS2-1 to obtain pSHCPRO-F. pCHCPRO-F was produced by inserting an *Sfi*I–*Sal*I DNA frag-

ment containing the HC-Pro sequence from pSHCPRO-F into the *Sfi*I, *Xho*I-digested pACT2.

*Plasmids for deletion mapping.* pCS422-S1 was made by inserting an *Nde*I fragment containing cDNA sequence from nt positions 1682–2302 of pCS4-22 into pAS2-1. The plasmid from which the *Nde*I fragment was deleted was religated to make pCS422-S3. A *Pst*I DNA fragment containing cDNA sequence from nt positions 3129–4799 was removed from pCS4-22 to construct pCS422-S2. A cDNA fragment (nt positions 1682–2338) was amplified from pCS4-22 with oligonucleotides GBT-1 (5′-TCATCGGAAGAGAGTAG-3′) and HCPRO-3. The amplified cDNA fragment was cleaved with *Eco*RI and ligated into the *Eco*RI, *Sma*I-digested pAS2-1 to produce pCS422-S5. Similarly, pSHCPRO-C was constructed by inserting an amplified cDNA fragment (nt positions 2102–2338) from pCS4-22 with oligonucleotides HCPRO-C (5′-GTGGATCCCTTGGCCAATGTTTGG-3′) and HCPRO-3 into pAS2-1. The cDNA fragment corresponding to the $NH_2$-terminal region of CI (nt positions 3329–3959) was amplified from pCS10-1 with oligonucleotides CI-5 and GAD-2 (5′-GTATCTACGATTCATAGATC-3′), digested with *Bam*HI, and ligated with *Bam*HI-digested pACT2 to make pCCI-N. pCS413-CIC, a deletion derivative of pCS4-13, was made by inserting the CI portion of pCS4-13 amplified with oligonucleotides GAD-1 (5′-AATACCACT ACAATGGATG-3′) and CI-3 into *Sfi*I and *Bam*HI sites of pACT2. Likewise, NIb or NIa portions of pCS19-2 and pCS19-11 amplified by PCR with appropriate oligonucleotides were inserted into pAS2-1 or pACT2 to produce pCS192-NIb and pCS1911-NIa, respectively. The cDNA inserts of pCS4-22 and pCS19-2 were excised with *Sfi*I and *Sal*I and inserted between the *Sfi*I and *Xho*I sites of pACT2 to construct pCS422-C1 and pCS192-C1, respectively. Analogously, pCS422-C5 was made by inserting the *Sfi*I–*Sal*I fragment of pCS422-S5 containing the COOH-terminal half of HC-Pro into pACT2 digested with *Sfi*I and *Xho*I.

*Plasmids for GST fusion proteins. Sma*I–*Eco*RI DNA fragments containing CI, CP, P1, or P3 cDNA sequences were isolated from pCCI-F, pCCP-F, pCP1-F, and pCP3-F. pGEX-CI, pGEX-CP, pGEX-P1, and pGEX-P3 were constructed by ligating the CI, CP, P1, or P3 cDNA fragments into a GST fusion protein expression vector, pGEX-2T (Pharmacia Biotech), respectively. An *Sma*I–*Bgl*II fragment containing the entire HC-Pro cDNA sequence was isolated from pCHCPRO-F. The 3′-*Bgl*II terminus was filled in before ligating into the *Sma*I site of pGEX-2T to produce pGEX-HCPRO.

## Immunoblot analyses

Yeast cells were grown in appropriate selective SD to reach mid-log phase ($OD_{600}$ of 0.6–0.8). Total proteins from yeast were prepared as described in Atkin *et al.* (1995) by breaking yeast cells with glass beads (0.4–0.6 mm) in ice-cold lysis buffer (5 mM EDTA, 250 mM NaCl, 0.1% Nonidet P-40, 50 mM Tris, pH 7.4) supplemented with protease inhibitors. Protein samples (10–20 $\mu$g) were fractionated on SDS–12% polyacrylamide gel, transferred to nitrocellulose membranes, and subjected to immunoblot analyses. Bound proteins were probed with antibodies against GAL4 AD or BD (1:200 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), and then with alkaline phosphatase-conjugated goat anti-rabbit IgG (1:2500 dilution; Bio-Rad, Hercules, CA).

## Expression of GST fusion proteins

*E. coli* XL2-Blue (Stratagene) harboring GST fusion protein plasmids were grown at 37°C overnight. Then 2 ml of overnight culture was inoculated into 200 ml of fresh media and grown to an optical density at 600 nm of 0.6–0.8. Expression of GST fusion proteins was induced by the addition of 0.5 mM isopropyl-$\beta$-D-thiogalactopyranoside for 2 h at 37°C. Cells were harvested and washed with 12 ml of STE (10 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA). Cells were resuspended with 10 ml of STE containing 100 $\mu$g/ml lysozyme and then incubated on ice for 15 min. DTT was added to a final concentration of 5 mM, and cells were lysed by the addition of *N*-lauroylsarcosine to a final concentration of 1.5% (Frangioni and Neal, 1993). After disruption of bacterial cells by sonication and clarification by centrifugation at 10,000 × *g* for 5 min, Triton X-100 was added to the lysate at a final concentration of 2%. Then 500 ml of 50% (v/v) glutathione–Sepharose 4B (Pharmacia Biotech) in PBS (140 mM NaCl, 2.7 mM KCl, 10.1 mM $Na_2HPO_4$, 1.8 mM $KH_2PO_4$, pH 7.3) was added to the bacterial lysate and incubated at room temperature for 30 min with gentle agitation. The Sepharose beads were washed eight times with PBS and finally resuspended with 250 $\mu$l of storage buffer (50 mM HEPES, pH 7.3, 150 mM NaCl, 5 mM DTT, 10% glycerol). Storage of Sepharose beads with bound protein was at 4°C. Aliquots of GST-fusion proteins immobilized on Sepharose beads were quantified on SDS–12% polyacrylamide gels.

## *In vitro* transcription and translation

DNA templates for *in vitro* transcription were prepared by PCR from appropriate WSMV cDNA sequences in pACT2 with a T7 RNA polymerase promoter primer (5′-TAATACGACTCACTATAGGGAGACCACATGGATGATGTATATAACTATCTATTC-3′) and a universal 3′ primer for pACT2 (5′-GTATCTACGATTCATAGATC-3′) (Bai and Elledge, 1997). *In vitro* transcription and translation were performed in the presence of [$^{35}$S]methionine using the TNT T7 coupled reticulocyte lysate system (Promega) as recommended by the manufacturer. DNA template (1–2 $\mu$g) was added to a total volume of 50 $\mu$l of transcription/translation mixture and incubated at 30°C for 2 h. Trans-

lation products were analyzed by SDS–12% PAGE, followed by autoradiography.

### *In vitro* protein binding assay

The procedure for *in vitro* protein binding assay (Bouyac *et al.,* 1997) was modified as follows. The amount of proteins bound to glutathione–Sepharose was estimated by SDS–PAGE, followed by Coomassie staining. Sepharose beads bound to the appropriate GST fusion protein were diluted with glutathione–Sepharose beads, if necessary, to use a similar amount of immobilized proteins. A 20-$\mu$l bed volume of Sepharose beads was incubated with 25 $\mu$l of *in vitro* translation mixture in a total volume of 300 $\mu$l of binding buffer (50 mM Tris–HCl, pH 7.6, 100–250 mM NaCl, 0.1% Triton X-100) at 4°C for 3 h. Beads were washed four times with 1 ml of ice-cold binding buffer, resuspended in 20 $\mu$l of 2× loading buffer (100 mM Tris–HCl, pH 6.8, 200 mM DTT, 4% SDS, 0.2% bromphenal blue, 20% glycerol), boiled for 3 min, and analyzed by SDS–PAGE and autoradiography.

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