

HC-Pro Protein of *Potato Virus Y* Can Interact with Three *Arabidopsis* 20S Proteasome Subunits In Planta[▽]

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The multifunctional protein helper component proteinase (HC-Pro) is thought to interfere with the activity of the 20S proteasome; however, no sites of interaction have been identified for either protein. Here, we first show that the *Potato virus Y* (PVY) HC-Pro protein can interact with three *Arabidopsis* 20S proteasome subunits (PAA, PBB, and PBE), using a yeast two-hybrid system and the bimolecular fluorescence complement assay. In addition, yeast two-hybrid analysis of the interaction between several mutant subunits of the 20S proteasome and PVY HC-Pro confirmed that residues 81 to 140 of PAA, 1 to 80 of PBB, and 160 to 274 of PBE are necessary for binding PAA, PBB, and PBE to PVY HC-Pro, respectively. Deletion mutant analysis of PVY HC-Pro showed that the N terminus (residues 1 to 97) is necessary for its interaction with three *Arabidopsis* 20S proteasome subunits. The ability of HC-Pro to interact and interfere with the activity of the 20S proteasome may help explain the molecular basis of its multifunctional character.

The helper component proteinase (HC-Pro) of the genus *Potyvirus* is one of three viral proteinases that release the viral proteins needed for infection (27). Although HC-Pro has several functions in the viral cycle, including transmission via aphids, replication, and cell-to-cell and systemic virus movement (12, 32), little is known about the molecular mechanisms involved or the links between the protein's various activities (12). HC-Pro suppresses RNA silencing, which is regarded as an in vivo plant antiviral defense, but details of the molecular mechanism are lacking (1, 6, 23, 24, 28). Recently, it was discovered that HC-Pro of *Lettuce mosaic virus* (LMV) could interfere with the activity of the 20S proteasome, a key element of the ubiquitin/26S proteasome system (Ub/26S), which is also involved in the antiviral response (4).

Ub/26S is one of the most important proteolytic pathways in eukaryotes (11, 12, 15). The core element of this tightly regulated and highly specific system is the 26S proteasome, a high-molecular-mass complex consisting of a cylindrical 20S core protease capped on each end by a 19S regulatory particle (25, 26, 31, 33, 34). The central barrel-shaped 20S proteasome is composed of four stacked rings, including seven α subunits in the two outer rings and seven β subunits in the two inner rings, resulting in an overall configuration of $\alpha 1-\alpha 7/\beta 1-\beta 7//\beta 1-\beta 7/\alpha 1-\alpha 7$ (7, 30).

Ub/26S contributes significantly to plant development by affecting a wide range of processes, including embryogenesis, hormone signaling, and senescence (13, 25, 30). In addition, the proteasome can interfere with the translation of viral RNA and mRNA in virus-infected cells (3, 5, 8, 14, 16, 22). Ballut et

al. (5) provided the first evidence of RNA endonuclease activity associated with the plant proteasome. Their results, which showed that the RNAs of two plant viruses, *Tobacco mosaic virus* and LMV, could be hydrolyzed by the proteasome, suggested that the endonuclease of the 20S proteasome might play an antiviral role in vivo (5).

Research on animal viral proteins has revealed that viruses can direct or subvert Ub/26S by interacting with the core of the 20S proteasome or with its 19S regulatory particle. For example, the human immunodeficiency virus type 1 Tat protein inhibits the peptidase activity and assembly of the 26S proteasome by interacting with distinct proteasome subunits (α and β) (2, 21, 29), while the hepatitis B virus X protein affects the 26S proteasome by interacting with the PMAS7 subunit of the 20S proteasome and PSMC1 subunit, which is a component of the 19S regulatory particle (19, 20, 40). Otherwise, the sequence of a viral protein can also interrupt proteasome function; for instance, Gly-Ala repeats act as stop-transfer signals during proteasome substrate processing (39).

Ballut et al. (4) were the first to describe the ability of a plant viral protein to interfere with the activity of the proteasome. They showed that HC-Pro from LMV could bind the proteasome and inhibit its 20S endonuclease activity in vitro, while its proteolytic activity was either unchanged or slightly stimulated (4). However, the interaction of a plant viral protein with the subunits of the 20S proteasome has not been described.

Using a yeast two-hybrid assay and bimolecular fluorescence complement (BiFC) assay, we found that potato virus Y (PVY) HC-Pro could interact with the PAA, PBB, and PBE subunits of the *Arabidopsis* 20S proteasome in yeast and in tobacco epidermal cells. Furthermore, we analyzed the domains necessary for these interactions in order to elucidate the mode of interaction between HC-Pro and the 20S proteasome.

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TABLE 1. Primers used for cloning 14 *Arabidopsis* cDNAs

Primer	Sequence
PAAF.....	5'-CGATTTTCGGAAGAGAGTTGTTTC-3'
PAAR.....	5'-TCCCGACTGAATGTTCCA-3'
PABF.....	5'-AAGGAGCTATGGGAGAT-3'
PABR.....	5'-TGTAATGCCACGAAA-3'
PACF.....	5'-AAGAAGAACCGAACGAG-3'
PACR.....	5'-TACCAGGCGAATAAGAC-3'
PADF.....	5'-AGATGGCGAGATACGAT-3'
PADR.....	5'-ATCTGAGACCGCTACTTT-3'
PAEF.....	5'-CGTTGTGCGAAGGAC-3'
PAER.....	5'-TCAAAGACGACGCGATG-3'
PAFF.....	5'-CGACGACGAGTGAAGAA-3'
PAFR.....	5'-CACAAAGCTTACATTTC-3'
PAGF.....	5'-AATCAAGGAAGATGAGTAGC-3'
PAGR.....	5'-CATCAAACGACGCAAT-3'
PBAF.....	5'-CATCAGTCATCACCGGAATC-3'
PBAR.....	5'-GAAAGATCGGGTCAATTC-3'
PBBF.....	5'-AAAATGTCGCGAGTCTTCC-3'
PBBR.....	5'-GAAATGGAGGCAACTACG-3'
PBCF.....	5'-CATCTTGCGCTTGGCTT-3'
PBCR.....	5'-ATCATCCATCCTTCCCT-3'
PBDF.....	5'-TCTGTAGACGGAAGATGGAGT-3'
PBDR.....	5'-ATCCTTTACGGAAGTACGC-3'
PBEF.....	5'-TTTCTCCAGTAAGGGTTT-3'
PBER.....	5'-AAGGCGATGAACGATTAG-3'
PBBF.....	5'-TAAGCCCTAGCTCAAA-3'
PBFR.....	5'-GATAAGGTTGGCTAAATG-3'
PBGF.....	5'-GAAGAACCTAAAGCTCACTACGATA-3'
PBGR.....	5'-AGCCGATGTGGTTGCTTGG-3'

MATERIALS AND METHODS

Plant materials and growth conditions. *Arabidopsis thaliana* ecotype Columbia was used in this study. Surface-sterilized seeds were sown on Murashige-Skoog medium, placed at 4°C in darkness for 4 days, and then allowed to germinate. All seedlings transferred to soil were grown under standard greenhouse conditions.

Cloning and identification of the 20S proteasome cDNAs from *A. thaliana*. The 14 cDNAs encoding the *Arabidopsis* 20S proteasome subunits included in this work are *PAA2* (GenBank accession no. AF043519), *PAB1* (AF043520), *PAC1* (AF043521), *PAD1* (AF043522), *PAE1* (AF043524), *PAF1* (AF043526), *PBA1* (AF043529), *PBB2* (AF043531), *PBC2* (AF043533), *PBD1* (AF043537), *PBE1* (AF043536), *PBF1* (AF043537), and *PBG1* (AF043538) (9); we designated the *Arabidopsis* 20S proteasome α and β subunits PAA-PAG and PBA-PBG as per Fu et al. (9). The primers used to clone these cDNAs are listed in Table 1. All cDNAs were cloned into pMD18-T (Takara) and verified by sequencing.

Yeast two-hybrid assay. The full-length coding sequence of PVY HC-Pro, which was fused to the GAL4 DNA-binding domain, was amplified by PCR from PVY N strain cDNA using the primers HC-Pro5 and HC-Pro3. The product was then cloned into pGBKT7 via BamHI/PstI digestion to form pGBKT7-HC-Pro. The full-length coding sequences of 14 *Arabidopsis* 20S proteasome subunits, which were fused to the GAL4 DNA-binding domain, were cloned into pGADT7. The primers and restriction sites used to create these constructs are listed in Table 2. Each of the 14 plasmids carrying a 20S proteasome subunit was separately cotransformed with pGBKT7-HC-Pro into *Saccharomyces cerevisiae* AH109 cells as described in the BD Matchmaker library construction and screening kit user manual. At the same time, the two plasmids supplied with the kits, pGBKT7-53 and pGADT7-RecT, were cotransformed as positive controls, while pGBKT7-HC-Pro and pGADT7 were cotransformed as negative controls.

BiFC assay. The vectors used in the BiFC assay (pUC-SPYNE, pUC-SPYCE, pSPYNE-35S, and pSPYCE-35S) were gifts from Klaus Harter and Jörg Kudla (36). The full-length coding sequence of PVY HC-Pro was cloned into pUC-SPYNE and pSPYNE-35S to create a fusion with the N-terminal fragment of yellow fluorescent protein (YFP) (pUC-SPYNE-HC-Pro and pSPYNE-35S-HC-Pro). The full-length coding sequences of three *Arabidopsis* 20S proteasome subunits (PAA, PBB, and PBE) were individually cloned into pUC-SPYCE and pSPYCE-35S to create a fusion with the C-terminal fragment of YFP. The primers and restriction sites used to construct these plasmids are listed in Table 2.

Two plasmids at a time were used to bombard onion (*Allium cepa*) epidermal

cells by the method of Chen et al. (38), and YFP fluorescence was assessed 12 to 18 h later (38). The relevant negative controls were produced at the same time. For infiltration of *Nicotiana benthamiana*, *Agrobacterium tumefaciens* strain EHA105 was used to infiltrate the abaxial air space of 2- to 4-week-old plants; the epidermal cell layers of the leaves were assayed for fluorescence 2 days later (35, 37).

Fluorescence analysis was performed on a Nikon Eclipse TE2000-E inverted fluorescence microscope equipped with a Nikon D-Eclipse C1 spectral confocal laser scanning system. YFP fluorescence was examined at 514 nm (excitation) using an argon laser with an emission band of 515 to 530 nm and 650 nm (chlorophyll autofluorescence).

Construction of deletion mutants for PAA, PBB, PBE, and HC-Pro. Deletion mutants were designed for each subunit that was determined to interact with the PVY HC-Pro protein: PAA1 (residues 81 to 246), PAA2 (residues 1 to 140), and PAA3 (residues 1 to 80) for PAA, PBB1 (residues 81 to 274), and PBB2 (residues 1 to 210) for PBB, and PBE1 (residues 81 to 274) and PBE2 (residues 1 to 159) for PBE. The coding sequences of these mutants were amplified individually by PCR using pGADT7-PAA, pGADT7-PBB, and pGADT7-PBE as the templates and the primers (PAA1-5, PAA1-3; PAA2-5, PAA2-3; PAA3-5, PAA3-3; PBB1-5, PBB1-3; PBB2-5, PBB2-3; PBE1-5, PBE1-3; and PBE2-5, PBE2-3) listed in Table 2. The products were then individually subcloned into pGADT7 and cotransformed with pGBKT7-HC-Pro into *S. cerevisiae* AH109 cells to determine which domains of the subunits were necessary for interacting with PVY HC-Pro.

Three deletion mutants were designed for PVY HC-Pro, HC-Pro1 (residues 98 to 456), HC-Pro2 (residues 1 to 298), and HC-Pro3 (residues 1 to 97). The coding sequences of these mutants were amplified individually by PCR using pGBKT7-HC-Pro as the templates and the primers (HC-Pro1-5, HC-Pro1-3; HC-Pro2-5, HC-Pro2-3; and HC-Pro3-5, HC-Pro3-3) listed in Table 2. The products were then individually subcloned into pGBKT7 and cotransformed with pGADT7-PAA, pGADT7-PBB, and pGADT7-PBE into *S. cerevisiae* AH109 cells to determine the necessary domain for interacting with PAA, PBB, and PBE.

RESULTS

Interaction of PVY HC-Pro with *Arabidopsis* 20S proteasome subunits in the yeast two-hybrid assay. To test which subunit of the *A. thaliana* 20S proteasome could interact with PVY HC-Pro, we cloned 14 cDNAs encoding the subunits of the *Arabidopsis* 20S proteasome and subcloned them into pGADT7. All clones were verified by sequencing; the predicted primary sequences we obtained were consistent with those described by Fu et al. (9). Each of the 14 plasmids encoding a subunit of the 20S proteasome was then cotransformed with pGBKT7-HC-Pro into *S. cerevisiae* AH109 cells. The transformants were cultivated on synthetic dropout (SD) medium lacking Leu and Trp (SD/-Leu/-Trp), followed by selection on SD medium lacking Ade, His, Leu, and Trp (SD/-Ade/-His/-Leu/-Trp).

Our yeast two-hybrid results indicate that three *Arabidopsis* proteasome subunits, PAA, PBB, and PBE, are capable of interacting with HC-Pro in *S. cerevisiae* AH109 cells. In other words, the DNA-binding domain and activation domain were brought into sufficient proximity to drive the transcription of the reporter genes (*ADE2*, *HIS3*, *lacZ*, and *MEL1*) that allowed the yeast to grow on SD/-Ade/-His/-Leu/-Trp (Fig. 1). Subunits that did not interact with HC-Pro could not restore auxotrophy in the yeast cells (i.e., the transformants were able to grow on SD/-Leu/-Trp but not on SD/-Ade/-His/-Leu/-Trp) (Fig. 1). pGBKT7-53 and pGADT7-RecT were cotransformed as positive controls, while pGBKT7-HC-Pro and pGADT7 were cotransformed as negative controls; the transformants were selected on SD/-Ade/-His/-Leu/-Trp.

Identification of the interaction between PVY HC-Pro and PAA, PBB, and PBE in living plant cells using BiFC. To verify

TABLE 2. Primers used in plasmid construction

Primer	Sequence	Restriction enzyme	Construct
HC-Pro5	5'-TAGGATTCTGTGCAATGCCGACAATTTT-3'	BamHI	pGBKT7-HC-Pro
HC-Pro3	5'-TACTGCAGACCAACTCTATAATGTTT-3'	PstI	
PAA5	5'-ATGAATTCATGAGCAGAGGAAGCGGC-3'	EcoRI	pGADT7-PAA
PAA3	5'-ATCTCGAGTCAGTCACGTTCACTAAT-3'	XhoI	
PAB5	5'-ATGAATTCATGGGAGATAGTCAGTAC-3'	EcoRI	pGADT7-PAB
PAB3	5'-ATCTCGAGTTACTCGACTTCAGCAAG-3'	XhoI	
PAC5	5'-ATGAATTCATGTCTCGGAGATATGAT-3'	EcoRI	pGADT7-PAC
PAC3	5'-ATGAGCTCTTAAGATGTTTCAGCAGC-3'	SacI	
PAD5	5'-ATGAATTCATGGCGAGATACGATCGA-3'	EcoRI	pGADT7-PAD
PAD3	5'-ATCTCGAGTCATGTTTCCTTCGCAGG-3'	XhoI	
PAE5	5'-ATGAATTCATGTTTCTCACTAGAACT-3'	EcoRI	pGADT7-PAE
PAE3	5'-ATCTCGAGTCAAAGACGAGCATGAC-3'	XhoI	
PAF5	5'-ATGAATTCATGTTTCAGAAACCAATAC-3'	EcoRI	pGADT7-PAF
PAF3	5'-ATCTCGAGTTACATTTCCATTGGAGC-3'	XhoI	
PAG5	5'-ATGAATTCATGAGTAGCATTGGAAC-3'	EcoRI	pGADT7-PAG
PAG3	5'-ATCTCGAGTTAGTCAGCATCCATCTC-3'	XhoI	
PBA5	5'-ATGAATTCATGGATCTCAATCTCGAT-3'	EcoRI	pGADT7-PBA
PBA3	5'-ATCTCGAGTCACATGGCCATTGGTTC-3'	XhoI	
PBB5	5'-ATGAATTCATGTCGAGTCTTCCGTC-3'	EcoRI	pGADT7-PBB
PBB3	5'-ATCTGCAGTCATTCTCCATGAGTTC-3'	XhoI	
PBC5	5'-ATGAATTCATGTCGATCTTCGAGTAC-3'	EcoRI	pGADT7-PBC
PBC3	5'-ATCTCGAGTCAATCCATCCTTCCCTT-3'	XhoI	
PBD5	5'-ATGAATTCATGGAGTGTGTATTCCGGT-3'	EcoRI	pGADT7-PBD
PBD3	5'-ATCTCGAGTCAGACAACAGCAGTTGT-3'	XhoI	
PBE5	5'-ATGAATTCATGAAGCTTGATACTAGT-3'	EcoRI	pGADT7-PBE
PBE3	5'-ATCTGCAGTTATTCGGCTGTTGCTTC-3'	SpeI	
PBF5	5'-ATGAATTCATGACTAAACAGCACGCG-3'	EcoRI	pGADT7-PBF
PBF3	5'-ATCTCGAGTTAGTCTTTCCTCAGGTC-3'	XhoI	
PBG5	5'-ATATCGATACATGACGACTTTTTCTG-3'	Clal	pGADT7-PBG
PBG3	5'-ATCTCGAGTTACCAGGAGCCTGCAGC-3'	XhoI	
HcProBiFC5	5'-ATTCTAGAATGTGCAATGCCGACAATTT-3'	XbaI	pUC-SPYCE-HC-Pro/pSPYNE-35S-HC-Pro
HcProBiFC3	5'-ATACTAGTACCAACTCTATAATGTTTTA-3'	SpeI	
BIFCPAA5	5'-ATTCTAGAATGAGCAGAGGAAGCGGCGC-3'	XbaI	
BIFCPAA3-1	5'-TAGGTACCGTCACGTTCACTAATGGCCG-3'	KpnI	pUC-SPYCE-PAA/pSPYCE-35S-PAA
BIFCPAA3-2	5'-TACTCGAGGTCACGTTCACTAATGGCCG-3'	XhoI	
BIFCPBB5	5'-ATTCTAGAATGTCGAGTCTTCCGTC-3'	XbaI	
BIFCPBB3-1	5'-ATGGTACCTTCTCCATAGCTTCACC-3'	KpnI	pUC-SPYCE-PBB/pSPYCE-35S-PBB
BIFCPBB3-2	5'-ATCCCGGGTCTCCATAGCTTCACC-3'	XmaI	
BIFCPBE5	5'-ATTCTAGAATGAAGCTTGATACTAGTGG-3'	XbaI	
BIFCPBE3-1	5'-TAGGTACCTTCGGCTGTTGCTTCTCCA-3'	KpnI	pUC-SPYCE-PBE/pSPYCE-35S-PBE
BIFCPBE3-2	5'-TAGGTACCTTCGGCTGTTGCTTCTCCA-3'	XmaI	
PAA1-5	5'-TAGAATTCATGACTGGCATGACAGCTGATTC-3'	EcoRI	pGADT7-PAA1
PAA1-3	5'-ATCTCGAGTCAGTCACGTTCACTAATGGCCG-3'	XhoI	
PAA2-5	5'-ATGAATTCATGAGCAGAGGAAGCGGCGCTGG-3'	EcoRI	pGADT7-PAA2
PAA2-3	5'-ATCTCGAGTCAAACCATGGCACTACTCCAA-3'	XhoI	
PAA3-3	5'-TACTCGAGTTAGGCTAACAATCCAAGGT-3'	XhoI	pGADT7-PAA3
PBB1-5	5'-TAGAATTCATGTAAGTGTGTGGTGCAGGA-3'	EcoRI	pGADT7-PBB1
PBB1-3	5'-TAGAGCTCTCATTCTCCATAGCTTCACC-3'	SacI	
PBB2-5	5'-TAGAATTCATGTCGAGTCTTCCGTC-3'	EcoRI	pGADT7-PBB2
PBB2-3	5'-TAGAGCTCTCAGCTACCACTACCAAG-3'	SacI	
PBE1-5	5'-TAGAATTCATGGGATATATCTCTCACAATC-3'	EcoRI	pGADT7-PBE1
PBE1-3	5'-ATGAGCTCTTATTCGGCTGTTGCTTCTCCA-3'	SacI	
PBE2-5	5'-ATGAATTCATGAAGCTTGATACTAGTGGGT-3'	EcoRI	pGADT7-PBE2
PBE2-3	5'-ATGAGCTCTTATCCAGCAATCATTGTACCAA-3'	SacI	
HC-Pro1-5	5'-TAGAATTCATGATAGCGTTAGAGCATCTA-3'	EcoRI	pGBKT7-HC-Pro1
HC-Pro1-3	5'-TACTGCAGTTAACCAACTCTATAATGTTT-3'	PstI	
HC-Pro2-5	5'-TAGAATTCATGTCGAATGCCGACAATTT-3'	EcoRI	pGBKT7-HC-Pro2
HC-Pro2-3	5'-TACTGCAGTTAACCATCATCAAGTGTGT-3'	PstI	
HC-Pro3-3	5'-TACTGCAGTTACAAGAACTTATTAACATG-3'	PstI	pGBKT7-HC-Pro3

our yeast two-hybrid assay results, we analyzed the interaction between the three *Arabidopsis* 20S proteasome subunits (PAA, PBB, and PBE) and PVY HC-Pro by BiFC assay. In this approach, a fluorescent complex is formed when two nonfluorescent fragments of YFP are brought together by an interac-

tion between the proteins fused to the fragments (17, 18). We constructed vectors expressing PAA, PBB, and PBE fused to the last 86 amino acids of YFP and a vector expressing HC-Pro fused to the first 155 amino acids of YFP (36). The two transient-expression vectors were then used to cotransform onion

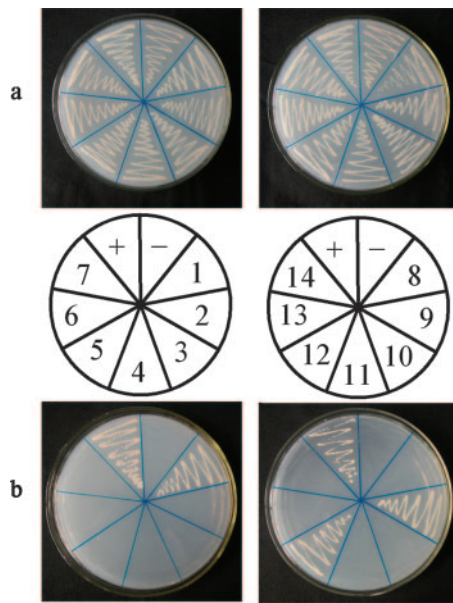


FIG. 1. Interaction of 14 *Arabidopsis* 20S proteasome subunits and PVY HC-Pro in transformed *S. cerevisiae* AH109 cells grown on SD/-Leu/-Trp (a) and on SD/-Ade/-His/-Leu/-Trp (b). Cotransformants of seven α subunits (left) and seven β subunits (right) (PAA to PAE) and HC-Pro are shown. +, pGBKT7-53/pGADT7-RecT (positive control); -, pGBKT7-HC-Pro/pGADT7 (negative control); 1, pGBKT7-HC-Pro/pGADT7-PAA; 2, pGBKT7-HC-Pro/pGADT7-PAB; 3, pGBKT7-HC-Pro/pGADT7-PAC; 4, pGBKT7-HC-Pro/pGADT7-PAD; 5, pGBKT7-HC-Pro/pGADT7-PAE; 6, pGBKT7-HC-Pro/pGADT7-PAF; 7, pGBKT7-HC-Pro/pGADT7-PAG; 8, pGBKT7-HC-Pro/pGADT7-PBA; 9, pGBKT7-HC-Pro/pGADT7-PBB; 10, pGBKT7-HC-Pro/pGADT7-PBC; 11, pGBKT7-HC-Pro/pGADT7-PBD; 12, pGBKT7-HC-Pro/pGADT7-PBE; 13, pGBKT7-HC-Pro/pGADT7-PBF; 14, pGBKT7-HC-Pro/pGADT7-PBG.

epidermal cells to test whether the proteins could interact, while the two constitutive vectors were individually transformed into *A. tumefaciens* strains EHA105 and EHA105 were used to coinfiltrate *N. benthamiana*.

As shown in Fig. 2, PAA, PBB, and PBE were able to interact with PVY HC-Pro in onion epidermal cells. YFP fluorescence was detected in the experimental samples 16 h after bombardment, whereas it was not detected in the negative control (i.e., pUC-SPYCE-HC-Pro and pUC-SPYNE [data not shown]). The assay in which *A. tumefaciens* was used to coinfiltrate *N. benthamiana* confirmed the interaction between PVY HC-Pro and PAA, PBB, and PBE in living plant cells (Fig. 3). Using the BiFC assay, we further confirmed that PVY HC-Pro could interact with these three *Arabidopsis* 20S proteasome subunits in living plant cells.

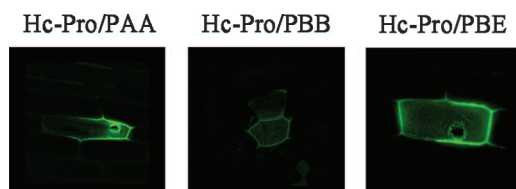


FIG. 2. Fluorescence of reconstructed YFP complexes (green) in onion (*A. cepa*) epidermal cells.

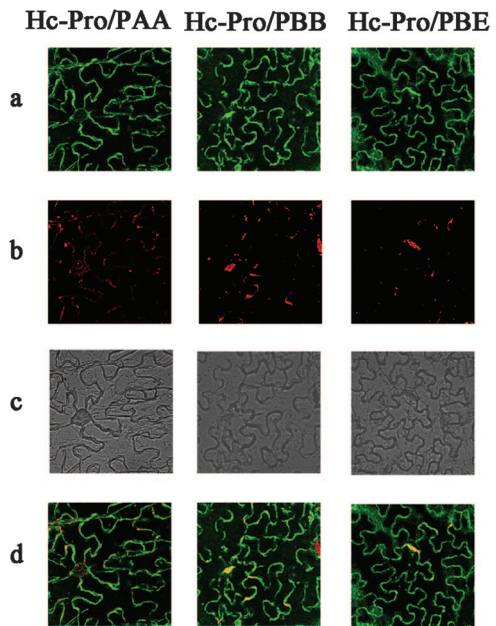


FIG. 3. Subcellular localization of reconstructed YFP complexes determined in leaf epidermis of *N. benthamiana*. a, YFP fluorescence (green); b, chlorophyll autofluorescence (red); c, bright field; d, YFP-chlorophyll autofluorescence overlay.

Identification of the necessary domains of PAA, PBB, and PBE for interacting with PVY HC-Pro. To determine the domains necessary for the interaction between HC-Pro and PAA, PBB, and PBE, we designed three deletion mutants for PAA and two deletion mutants for PBB and PBE (Fig. 4). The coding sequences of the mutants were subcloned into pGADT7 and separately cotransformed with pGBKT7-HC-Pro into *S. cerevisiae* AH109 cells. The domains required for the interaction were subsequently identified by a yeast two-hybrid assay.

As shown in Fig. 5, the vectors expressing PAA1 (residues 81 to 246), PAA2 (residues 1 to 140), PBB2 (residues 1 to 210), and PBE1 (residues 81 to 274), which were each cotransformed with pGBKT7-HC-Pro into *S. cerevisiae* AH109 cells, restored the auxotrophy of the yeast cells, such that the transformants were able to grow on SD/-Ade/-His/-Leu/-Trp. In comparison, the transformants expressing PAA3 (residues 1 to 80), PBB1 (residues 81 to 274), or PBE2 (residues 1 to 159) with pGBKT7-HC-Pro were able to grow on SD/-Leu/-Trp but not on SD/-Ade/-His/-Leu/-Trp (Fig. 5).

These results indicate that the binding activities of the deletion mutants PAA1, PAA2, PBB2, and PBE1 were either similar to or greater than those of the wild-type proteins; in contrast, PAA3, PBB1, and PBE2 did not bind PVY HC-Pro. From these data, we conclude that residues 1 to 80 and 160 to 274 are necessary for the binding of PBB and PBE to PVY HC-Pro, respectively, because the deletion of these regions affected the interaction. Residues 81 to 140 of PAA may be the necessary domain for binding to PVY HC-Pro, because the deletion of the N terminus (residues 1 to 80) or the C terminus (residues 141 to 246) of PAA did not affect the interaction between PAA and PVY HC-Pro. In addition, the deletion mutant PAA1 (residues 1 to 80), which includes a conserved

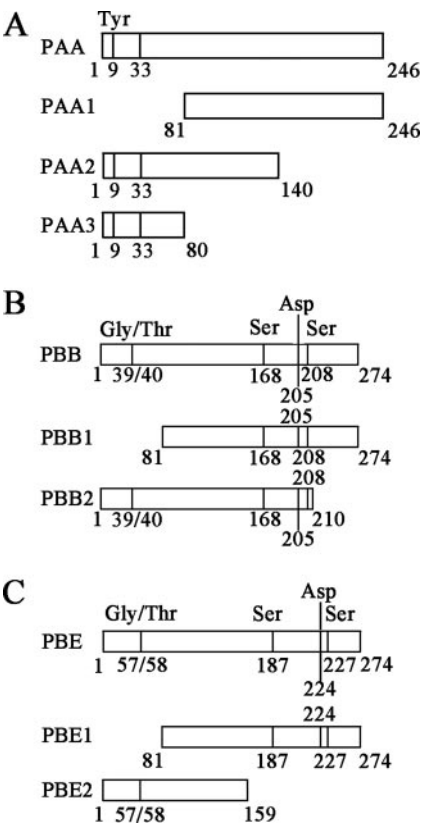


FIG. 4. Schematic overview of domains and deletion mutants of PAA (A), PBB (B), and PBE (C).

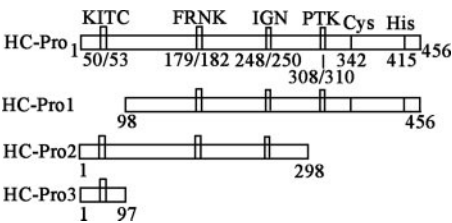


FIG. 6. Schematic overview of domains and deletion mutants of PVY HC-Pro.

domain (residues 9 to 33), could not bind to PVY HC-Pro and bring the DNA-binding domain and activation domain into sufficient proximity to drive the transcription of the reporter genes (*ADE2*, *HIS3*, *lacZ*, and *MEL1*) that allowed the yeast to grow on SD/–Ade/–His/–Leu/–Trp (Fig. 5).

Identification of the necessary domain of PVY HC-Pro for interacting with PAA, PBB, and PBE. HC-Pro can be schematically divided into three regions: an N-terminal region, a C-terminal region, and a central region (27). In order to test which region is necessary for the binding to 20S proteasome subunits, we constructed three deletion mutants of PVY HC-Pro: HC-Pro1 (residues 98 to 456), HC-Pro2 (residues 1 to 298), and HC-Pro3 (residues 1 to 97) (Fig. 6). The domains required for the interaction were subsequently identified by a yeast two-hybrid assay.

As shown in Fig. 7, HC-Pro2 (residues 1 to 298) and HC-Pro3 (residues 1 to 97) could individually bind to PAA, PBB, and PBE and then drive the transcription of the reporter genes (*ADE2*, *HIS3*, *lacZ*, and *MEL1*) and restore the auxotrophy of the yeast cells, so that the transformants were able to grow on

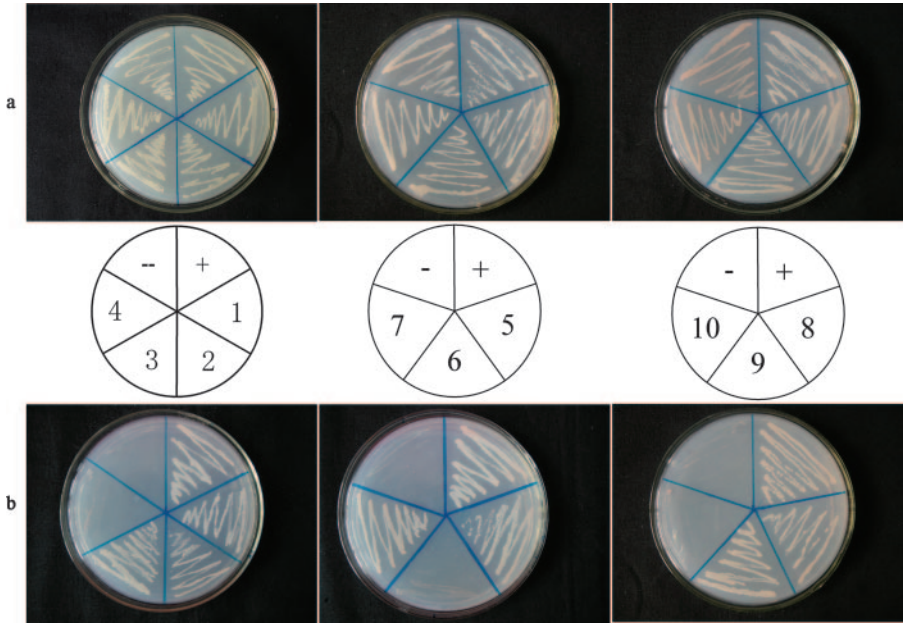


FIG. 5. Interaction of PVY HC-Pro and mutants of PAA (left), PBB (middle), and PBE (right) in transformed *S. cerevisiae* AH109 cells grown on SD/–Leu/–Trp (a) and on SD/–Ade/–His/–Leu/–Trp (b). +, pGBKT7-53/pGADT7-RecT (positive control); –, pGBKT7-HC-Pro/pGADT7 (negative control); 1, pGBKT7-HC-Pro/pGADT7-PAA; 2, pGBKT7-HC-Pro/pGADT7-PAA1; 3, pGBKT7-HC-Pro/pGADT7-PAA2; 4, pGBKT7-HC-Pro/pGADT7-PAA3; 5, pGBKT7-HC-Pro/pGADT7-PBB; 6, pGBKT7-HC-Pro/pGADT7-PBB1; 7, pGBKT7-HC-Pro/pGADT7-PBB2; 8, pGBKT7-HC-Pro/pGADT7-PBE; 9, pGBKT7-HC-Pro/pGADT7-PBE1; 10, pGBKT7-HC-Pro/pGADT7-PBE2.

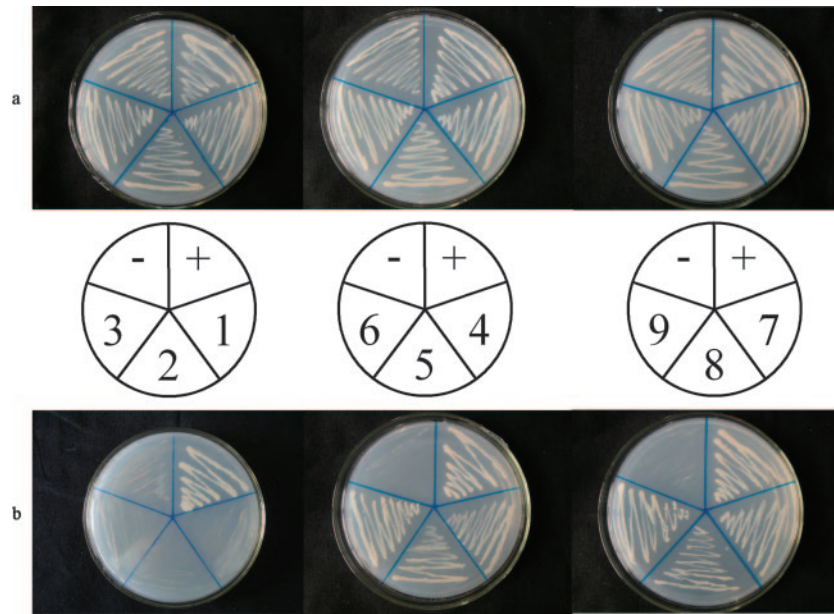


FIG. 7. Interaction of mutants of PVY HC-Pro and PAA, PBB, and PBE in transformed *S. cerevisiae* AH109 cells grown on SD/-Leu/-Trp (a) and on SD/-Ade/-His/-Leu/-Trp (b). Cotransformants of HC-Pro1 (left), HC-Pro2 (middle), and HC-Pro3 (right) and PAA, PBB, and PBE are shown. +, pGBKT7-53/pGADT7-RecT (positive control); -, pGBKT7-HC-Pro/pGADT7 (negative control); 1, pGBKT7-HC-Pro1/pGADT7-PAA; 2, pGBKT7-HC-Pro1/pGADT7-PBB; 3, pGBKT7-HC-Pro1/pGADT7-PBE; 4, pGBKT7-HC-Pro2/pGADT7-PAA; 5, pGBKT7-HC-Pro2/pGADT7-PBB; 6, pGBKT7-HC-Pro2/pGADT7-PBE; 7, pGBKT7-HC-Pro3/pGADT7-PAA; 8, pGBKT7-HC-Pro3/pGADT7-PBB; 9, pGBKT7-HC-Pro3/pGADT7-PBE.

SD/-Ade/-His/-Leu/-Trp. However, HC-Pro1 (residues 98 to 456) did not interact with PAA, PBB, and PBE. From these results, we confirmed that the N terminus of PVY HC-Pro plays an important role in the binding to PAA, PBB, and PBE, because the deletion of this region affected the interaction between PVY HC-Pro and PAA, PBB, and PBE, and the N-terminal region of PVY HC-Pro (residues 1 to 97) itself interacted with PAA, PBB, and PBE.

DISCUSSION

HC-Pro is vital for the transmission of PVY, but the exact mechanisms underlying its multifunctional character are unclear. Recently, Ballut et al. (5) reported that HC-Pro from LMV was able to bind to the 20S proteasome and affect its activity; however, exactly which subunits of the 20S proteasome are bound by HC-Pro is unknown. In this report, we present the first evidence that PVY HC-Pro interacts with three *Arabidopsis* 20S proteasome subunits, PAA, PBB, and PBE, using yeast two-hybrid and BiFC assays, which allow protein interactions to be examined under conditions that mimic the normal physiological environment (36). Our results demonstrate that PVY HC-Pro binds to distinct (α and β) subunits instead of binding one site on the *Arabidopsis* 20S proteasome.

The human proteasome 20S complex subunit Zeta harbors endonuclease activity (10); correspondingly, subunit PAE of the *Arabidopsis* 20S proteasome may also harbor this activity, especially since the *Arabidopsis* 20S proteasome α and β subunits have greater similarity to specific subunits in yeast and other organisms than with other α and β subunits in *Arabidopsis* (9). Recently, HC-Pro of LMV was reported to inhibit the endonuclease activity of the 20S proteasome in

vitro, and the authors suggested that HC-Pro affects RNase activity directly rather than indirectly through protection of the template (4). According to our results, PVY HC-Pro did not directly bind to the subunit harboring the RNase activity; instead, it bound to another α subunit, PAA (Fig. 1, 2, and 3). We therefore conclude that HC-Pro may indirectly inhibit the endonuclease activity of the 20S proteasome by binding PAA. In addition, an analysis of the primary sequence of PAA revealed the existence of a conserved region near the N terminus (residues 9 to 33) (Fig. 4A). In the *T. acidophilum*-yeast complex, this region assumes an α -helical structure, which is necessary for the assembly and/or subsequent stabilization of specific α -subunit- α -subunit contacts, and the conserved Tyr9 residue in the N terminus of PAA plays a crucial role in this interaction (9). Using deletion mutants of PAA, we found that PVY HC-Pro might bind residues 81 to 140 of PAA but not the conserved region near the N terminus (Fig. 5); that is, the function of this conserved domain in PAA, like that in the *T. acidophilum*/yeast complex, may be either unaffected or indirectly affected.

We also found that PVY HC-Pro could interact with PBB and PBE of the *Arabidopsis* 20S proteasome (Fig. 1, Fig. 2, and Fig. 3). Based on sequence alignments with comparable *T. acidophilum* and yeast subunits, it has been suggested that PBB and PBE may be involved in forming protease active sites (9). Additional analysis of these two subunits revealed that Gly39/Thr40 in PBB and Gly57/Thr58 in PBE are cleaved to liberate the Thr active site (Fig. 4). In addition, Ser168, Asp205, and Ser208 in PBB (Fig. 4B) and Ser187, Asp224, and Ser227 in PBE (Fig. 4C) may be involved in forming the catalytic site, since in the three-dimensional complex they may be adjacent

to the Thr in the active site. Our analysis of the interaction between PVY HC-Pro and several mutants of PBB and PBE indicates that HC-Pro binds residues 1 to 80 of PBB, which includes the conserved residues Gly39/Thr40, and residues 160 to 274 of PBE, which includes the conserved residues Ser187, Asp224, and Ser227 (Fig. 5). Thus, the domains of PBB and PBE that are necessary for binding to HC-Pro do not contain the same conserved residues. This indicates that HC-Pro does not directly bind these conserved residues, and it may not affect active-site formation in the protease.

We schematically divided PVY HC-Pro into three regions: an N-terminal region (residues 1 to 97), a central region (residues 98 to 298), and a C-terminal region (residues 299 to 456). Yeast two-hybrid analysis showed that the N-terminal region of PVY HC-Pro was necessary for the interaction with PAA, PBB, and PBE. The N-terminal region of HC-Pro was found to be involved only in the virus transmission process. Our results suggest that the N-terminal region of HC-Pro might have some other function in the infective process; for example, it may play an important role in the interaction with host proteins. However, our results are contrary to those reported by Ballut et al. (4), and there are some possible explanations for this. One may be that we use the yeast two-hybrid assay to confirm the interaction *in vivo*, while they analyzed the interaction *in vitro*. Another may be that we analyzed the exact subunit that can interact with PVY HC-Pro, while they studied the interaction between the whole 20S proteasome complex and LMV HC-Pro.

Based on our results, we propose that PVY HC-Pro binds the outside of the barrel of the *Arabidopsis* 20S proteasome by binding to several residues in the PAA, PBB, and PBE subunits. This would explain previous results showing that HC-Pro from LMV inhibits the endonuclease activity but not the proteolytic activity of the 20S proteasome *in vitro* (4). However, we have not yet identified the exact binding sites or the mode of interaction between HC-Pro and the 20S proteasome. Crystal structure analysis of the interaction may help resolve these issues.

Ub/26S plays a critical role in controlling development by affecting a wide range of processes, whereas viruses alter several functional systems, including Ub/26S, in order to facilitate their entry into cells (19, 20, 29). Some viral proteins can interfere with the function of Ub/26S by interacting with certain key elements. For example, several viral proteins, such as Tat and hepatitis B virus X protein, can interact with the 26S proteasome by binding to distinct subunits in the complex (2, 40). Here, we confirm that a plant virus protein, PVY HC-Pro, can interact with the PAA, PBB, and PBE subunits of the *Arabidopsis* 20S proteasome. Therefore, we presume that the ability to bind the proteasome may be a common feature of viral proteins that affect Ub/26S. Additional studies of the interaction between PVY HC-Pro and the *Arabidopsis* 20S proteasome will help us elucidate the molecular mechanisms underlying the multifunctional character of this protein as well as the relationship between viruses and cells.

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