Plastocyanin Transit Peptide Interacts with *Potato virus X* Coat Protein, While Silencing of Plastocyanin Reduces Coat Protein Accumulation in Chloroplasts and Symptom Severity in Host Plants

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Potato virus X coat protein (PVXCP) is, through communication with host proteins, involved in processes such as virus movement and symptom development. Here, we report that PVXCP also interacts with the precursor of plastocyanin, a protein involved in photosynthesis, both in vitro and in vivo. Yeast two-hybrid analysis indicated that PVXCP interacted with only the plastocyanin transit peptide. In subsequent bimolecular fluorescence complementation assays, both proteins were collocated within chloroplasts. Western blot analyses of chloroplast fractions showed that PVXCP could be detected in the envelope, stroma, and lumen fractions. Transmission electron microscopy demonstrated that grana were dilated in PVX-infected *Nicotiana benthamiana*. Furthermore, virus-induced gene silencing of plastocyanin by prior infection of *N. benthamiana* using a *Tobacco rattle virus* vector reduced the severity of symptoms that developed following subsequent PVX infection as well as the accumulation of PVXCP in isolated chloroplasts. However, PVXCP could not be detected in pea chloroplasts in an in vitro re-uptake assay using the plastocyanin precursor protein. Taken together, these data suggest that PVXCP interacts with the plastocyanin precursor protein and that silencing the expression of this protein leads to reduced PVXCP accumulation in chloroplasts and ameliorates symptom severity in host plants.

The majority of chloroplast proteins are synthesized in the cytoplasm as precursors that are subsequently directed to these organelles by their N-terminal peptides (Ellis 1981; Schmidt and Mishkind 1986). Protein import across the chloroplast envelope requires the specific recognition of precursor proteins at the outer envelope and their subsequent translocation across the inner membranes (Gary and Row 1995; Kouranov and Schnell 1996; Schnell 1995). Upon entering the stromal compartment, the N-terminus of the transit peptide is removed by a stromal processing peptidase (Abad et al. 1989; Robinson and

Ellis 1984). The C-terminus of the transit peptide is also cleaved during or after translocation across the thylakoid membrane, thereby releasing the mature protein into the thylakoid lumen (Hageman et al. 1990). Plastocyanin, a soluble copper-containing protein present in the thylakoid lumen and involved in the electron transport chain of photosystem I, employs this precursor recognition and cleavage strategy to enter chloroplasts (Lawrence and Kindle 1997).

Numerous distinct interactions are known to occur between viral and chloroplast proteins. The interaction of *Tomato mosaic virus* coat protein (CP) with a long distance movement-related protein in tobacco, designated IP-L, and the colocalization of both proteins at the thylakoid membranes are believed to be processes central to the development of chlorotic symptoms in infected plants (Zhang et al. 2008). In a somewhat more complex manner, the role of the chloroplast protein NRIP1 in the activation of defense signaling is affected by direct interactions with both the plant N immune receptor and the helicase domain of *Tobacco mosaic virus* (TMV) (Caplan et al. 2008). Additionally, this viral domain may also interact with the 33K subunit of the oxygen-evolving complex of photosystem II, as infection of *Nicotiana benthamiana* with TMV results in a decrease in 33K subunit mRNA levels, while silencing of the 33K subunit protein appeared to enhance TMV replication (Abbink et al. 2002). Similarly, downregulation of the *psaK* gene of photosystem I leads to increased viral titres during *Plum pox virus* (PPV) infections, suggesting a negative effect of the gene product on virus replication (Jiménez et al. 2006), while the cylindrical inclusion body protein of PPV appears to both interact with and decrease the expression of the photosystem I protein PSI-K. Other examples of virus protein interactions with chloroplasts involve the HC-Pro proteins. The HC-Pro of *Potato virus Y*, for example, has been detected in the chloroplasts of infected leaves and has been shown to interact with the chloroplast division-related factor, NtMinD (Jin et al. 2007). The HC-Pro protein of *Sugar cane mosaic virus* likewise interacts with the precursor of ferredoxin-5 (Fd V) in maize, resulting in a significant downregulation of Fd V mRNA levels and, according to Cheng and associates (2008), may sequentially interfere with the import of Fd V into chloroplasts.

Potato virus X (PVX), the type member of the potexvirus group (Koenig and Lesemann 1989), has proven useful as a model system for studying various aspects of virus infection (Batten et al. 2003). The CP (PVXCP) has been variously shown

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Nucleotide sequence data for *Nicotiana benthamiana* plastocyanin is available in the GenBank database under accession number FJ598042.

*The e-Xtra logo stands for “electronic extra” and indicates that a supplementary figure is published online.

to be involved in the processes of virus replication, movement, and virion formation (Atabekov et al. 2001; Baratova et al. 1992, 2004; Chapman et al. 1992; Verchot-Lubicz et al. 2007). It is also responsible for the induction of Rx resistance in potato and tobacco (Baurès et al. 2008; Bendahmane et al. 1995; Goulden et al. 1993), and it plays a major role in regulating translational activity at different stages of the infection process (Atabekov et al. 2001; Zayakina et al. 2008). PVXCP has been previously shown to interact with the *N. benthamiana* susceptibility factor protein for PVX infection, NbPCIP1 (Park et al. 2009). These studies demonstrate the significance of PVXCP at all stages of the virus-host interaction process.

In the current work, we show the presence of PVXCP in chloroplasts as determined by organelle fractionation and transmission electron microscopy (TEM). The significance of this interaction on viral pathogenesis was examined using virus-induced gene silencing (VIGS). We employed the yeast two-hybrid system and co-immunoprecipitation (co-IP) to identify and confirm an interaction between PVXCP and plastocyanin. Bimolecular fluorescence complementation assays (BiFC) allowed the determination of the interaction site, while an in vitro import assay (Rensink et al. 1998) was conducted to evaluate whether PVXCP, facilitated only by the precursor protein of plastocyanin, could enter the chloroplasts. Our results suggest that PVXCP interacts with precursor plastocyanin in vivo and that downregulating plastocyanin expression results in a reduced accumulation of PVXCP in chloroplasts and ameliorated symptoms in the host plants.

RESULTS

PVXCP interacts with both the precursor protein and the transit peptide of *N. benthamiana* plastocyanin in yeast.

The predicted precursor protein of plastocyanin (a mature chain of 99 amino acids) contains a transit peptide of 70 amino acids. Alignment of the deduced amino acid sequence of precursor plastocyanin from *N. benthamiana* (GenBank accession number FJ598042) with those of several other plant species (Supplementary Fig. 1) identified substantial sequence identity, i.e., *Solanum lycopersicum* (84%), *S. commersonii* (81%), *Spinacia oleracea* (73%), *Pisum sativum* (71%), and *Arabidopsis thaliana* (68%).

We conducted yeast two-hybrid experiments using PVXCP as bait to confirm its interaction with the plastocyanin protein of *N. benthamiana*. Yeast expressing PVXCP and precursor plastocyanin were shown to grow on a medium lacking leucine (Fig. 1A, segment 3), thereby demonstrating a direct interaction between PVXCP and the precursor protein of plastocyanin. Yeast containing pGBKT7-53/pGADT7-RecT (Fig. 1A, segment 1) and pGBKT7/pGADT7 (Fig. 1A, segment 2) were used as positive and negative controls, respectively.

In order to identify the specific region of plastocyanin necessary for this interaction, individual constructs using the pGADT7 vector expressing either the plastocyanin transit peptide (PCTP) or mature plastocyanin (PCMP) were tested for an interaction with PVXCP expressed from the pGBKT7 vector. Whereas no interaction was observed in assays expressing PVXCP and PCMP (Fig. 1A, segment 4), positive and reproducible interactions were detected in yeast assays expressing PVXCP and PCTP (Fig. 1A, segment 5). These findings indicate that the region of plastocyanin involved in the interaction with PVXCP is located within the N-terminal fragment and is most probably associated with the transit peptide region.

PVXCP interacts with the precursor protein of plastocyanin in vivo.

The results obtained from our interaction studies using the yeast two-hybrid system prompted us to investigate whether such interaction also occurs in planta. Specific and reproducible co-immunoprecipitation of PVXCP from total protein extracts of PVX-infected tobacco leaves using an anti-plastocyanin antibody indicated that PVXCP and plastocyanin form a protein complex in PVX-infected whole plants (Fig 1B). Hence confirming, in whole plants, the interaction between PVXCP and plastocyanin we first demonstrated using yeast two-hybrid analysis.

PVXCP interacts with precursor plastocyanin and its transit peptide in tobacco protoplasts.

We used BiFC to verify the interaction of PVXCP with plastocyanin and to determine the subcellular location of both proteins within living cells. The transient coexpression of PVXCP (from the construct pUC-SPYNE-PVXCP) and plastocyanin

Fig. 1. Interaction of plastocyanin and *Potato virus X* coat protein (PVXCP) in vitro and in vivo. **A**, The interactions between plastocyanin and PVXCP in yeast. Segment 1, pGBKT7-53/pGADT7-RecT (positive control); segment 2, pGBKT7/pGADT7 (negative control); segment 3, pGBKT7-CP/pGADT7-PC; segment 4, pGBKT7-CP/pGADT7-PCMP; segment 5, pGBKT7-CP/pGADT7-PCTP. **B**, Coimmunoprecipitation of PVXCP and plastocyanin from PVX-infected leaves of *Nicotiana benthamiana*. 'Extract with PVX' shows the total protein extracted from PVX-infected leaves. 'Pre-immune' shows preimmune serum coupled to amine-reactive gel (negative control). 'Anti-PC' shows immunoprecipitates immobilized onto the amine-reactive gel coupled with anti-plastocyanin antibody. The samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis and were immunoblotted with antibodies against either PVX or plastocyanin (PC).

(from the construct pUC-SPYCE-PC) in tobacco protoplasts resulted in the detection, by confocal microscopy, of punctuate yellow fluorescence collocated with chlorophyll autofluorescence (Fig. 2, first row). The yellow fluorescence was also apparent and similarly collocated in cells transiently coexpressing PVXCP and the plastocyanin transit peptide alone (pUC-SPYCE-PCTP; Fig 2, second row). These results indicate that PVXCP and the plastocyanin transit peptide also interact in vivo. Further, the apparent subcellular distribution of the yellow fluorescent protein (YFP) complexes on the surface of or within the chloroplasts suggests that this could be the location of their interaction within the cells. No YFP signal was detected in controls coexpressing PVXCP and mature plastocyanin (pUC-SPYCE-PCMP), indicating that PVXCP cannot interact with the truncated plastocyanin protein (Fig. 2, third row). Pairwise expression of unfused YFP^N and YFP^C (Walter et al. 2004) did not generate YFP fluorescence in protoplasts (Fig. 2, bottom row). From these results, it can be concluded that the transit peptide of plastocyanin alone is involved in the interaction with PVXCP in plant cells.

Effects of PVX infection on plastocyanin mRNA levels and protein expression.

The expression levels of certain host factors that interact with viral proteins are altered following viral infection (Dufresne et al. 2008; Kim et al. 2008; Park et al. 2009). To study the ex-

pression pattern of the plastocyanin gene, leaf tissues from four-week-old *N. benthamiana* (mock and PVX-inoculated) were harvested 14 days postinoculation (dpi). Reverse transcription-polymerase chain reaction (RT-PCR) analysis quantifying the plastocyanin gene transcripts against an internal control (actin gene) allowed a relative determination of the expression levels of plastocyanin following PVX infection. Our results show no significant difference in the expression levels of plastocyanin in samples extracted from either mock-inoculated or PVX-infected plants (Fig. 3A). These results thus demonstrate that plastocyanin mRNA levels are not altered at 14 dpi in PVX-infected leaves.

In order to test whether the level of functional plastocyanin was altered following virus infection, we purified and fractionated chloroplasts from mock-inoculated and PVX-infected *N. benthamiana* leaves at 14 dpi. Proteins were extracted from the envelope, stroma, membrane, and lumen fractions, were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and were subjected to Western blot using an anti-plastocyanin antibody. The band intensities observed indicated that the accumulation of plastocyanin in the different compartments of the chloroplasts was not significantly altered in comparison to equivalent samples from uninfected controls (Fig. 3B). Therefore, PVX infection does not affect the level of expression of plastocyanin either at the mRNA or protein levels.

Fig. 2. *Potato virus X* coat protein (PVXCP) interacts with the precursor protein and transit peptide of plastocyanin in plant cells. Subcellular localization of reconstructed yellow fluorescent protein (YFP) complexes in protoplasts of *Nicotiana benthamiana* using bimolecular fluorescence complementation assays. pUC-SPYNE-CP = PVXCP fused to the YFP N-terminus. pUC-SPYCE-PC, pUC-SPYCE-PCTP, and pUC-SPYCE-PCMP represent, respectively, the entire plastocyanin protein, the transit peptide, and the mature peptide of plastocyanin fused to the YFP C-terminus. **A**, YFP fluorescence; **B**, chlorophyll autofluorescence; **C**, bright field; and **D**, YFP/chlorophyll autofluorescence overlay.

PVXCP enters chloroplasts post-translationally.

Chloroplasts were purified from PVX-infected *N. benthamiana* leaves and were fractionated by centrifugation prior to analysis of the extracted proteins by Western blot. PVXCP was found in multiple fractions (envelope, stroma, and lumen) but was absent from the membrane fraction (Fig. 4A).

To determine whether the PVXCP detected in the chloroplasts was translated within the organelles or was translocated post-translationally from the cytoplasmic compartment, we extracted total chloroplast RNA from mock-inoculated and PVX-infected leaves. These samples were subjected to RT-PCR, using the *RuBisCO* large subunit gene encoded by the chloroplast genome as an internal control to approximate the equal loading of total RNA for each sample. PVXCP RNA could not be detected in either the PVX or mock-inoculated samples (Fig. 4B), thereby demonstrating the fidelity of the system and allowing us to deduce that PVXCP is not translated in the chloroplasts but is expressed from the viral RNA in the cytoplasm and subsequently translocated into these organelles.

Disruptions to the structure of the chloroplasts are apparent in PVX-infected tissues.

To confirm the distribution of PVXCP in the chloroplasts indicated by our fractionation experiments, anti-PVX antibody and anti-rabbit immunoglobulin G (IgG) conjugated with 15-nm diameter gold particles were used to detect PVXCP on

ultrathin sections of virus-infected leaves, using TEM. Considerable numbers of immunogold particles were seen both in the stroma and on the thylakoid membranes of chloroplasts in PVX-infected leaves (Fig. 5A). Immunogold particles were rarely seen on negative controls incubated with preimmune serum (Fig. 5B). This result is consistent with the fractionation assays and provides confirmation that PVXCP is able to penetrate the inner compartments of chloroplasts.

The structural impact of PVX infection on the chloroplasts was determined following the contrasting observation of clearly defined thylakoid layers in healthy leaves (Fig. 5C) in comparison to the dilated granal lamella and the presence of thylakoid vesicles and enlarged stromal areas found in the chloroplasts of PVX-infected leaves (Fig. 5D).

Downregulation of plastocyanin expression decreases the accumulation of PVXCP in chloroplasts and ameliorates symptoms in whole plants.

In plastocyanin-silenced *N. benthamiana*, semiquantitative RT-PCR showed that plastocyanin RNA could only be detected at a low level, even after 30 cycles (Fig. 6A, upper left panel, S). Similarly, plastocyanin protein was undetectable by Western blot analysis of samples from the leaves of silenced plants (Fig. 6A, upper right panel). Silenced plants also exhibited more severe chlorosis (Fig. 6A, lower panel, S) than non-silenced *N. benthamiana* plants infected with (*Tobacco rattle virus*) TRV alone (Fig. 6A, lower panel, T).

To further illustrate an interaction with plastocyanin during PVX-infections, an upper leaf on the plastocyanin-silenced plants was challenge-inoculated with PVX. While PVX induced prominent mosaic symptoms at 5 dpi on the upper noninoculated leaves of nonsilenced TRV-infected control plants (Fig. 6B, T+PVX), symptoms of PVX infection appeared later (at 7 dpi) and were less severe on equivalent leaves taken from the plastocyanin-silenced plants (Fig. 6B, S+PVX). Lower levels

Fig. 3. The expression pattern of plastocyanin was unchanged after *Potato virus X* (PVX) infection. **A**, Real time reverse transcription-polymerase chain reaction (RT-PCR) analysis of plastocyanin gene expression in the upper leaves of mock-inoculated (Mock) and PVX-infected (PVX) *Nicotiana benthamiana*. The RT-PCR quantification method relied upon the calculation of amplification efficiency against the actin gene as an internal loading control. Three individual plants were pooled for each sample (Mock or PVX-infected). **B**, Western blot detection of plastocyanin from different chloroplast fractions. M indicates fractions of chloroplasts purified from mock-inoculated leaves. I indicates fractions of chloroplasts purified from PVX-infected leaves. PC = plastocyanin.

Fig. 4. *Potato virus X* coat protein (PVXCP) enters chloroplasts post-translationally. **A**, Western blot detection of PVXCP in the envelope, stroma, and lumen fractions. **B**, Reverse transcription-polymerase chain reaction amplification of the PVXCP gene to determine the presence or absence of PVX RNA in chloroplasts. Total RNA extracted from PVX-infected *Nicotiana benthamiana* leaves was used as a positive amplification control, and the *RuBisCO* large subunit gene (*RbcL*)—encoded by chloroplast RNA—was used as a loading control. M represents fractions of chloroplasts purified from mock-inoculated leaves. I represents fractions of chloroplasts purified from PVX-infected leaves.

of PVXCP were also detected in the envelope, stroma, and lumen of chloroplasts isolated from silenced plants at 30 dpi (Fig. 6C), whereas the level of PVXCP in total leaf protein extracts remained unchanged (Fig. 6C, last two lanes on the right). Taken together, these findings indicate that silencing of plastocyanin does not affect the accumulation of PVXCP in whole plants but does impact the distribution of PVXCP in the chloroplastic compartments. We speculate that silencing of plastocyanin affects either the accumulation or translocation or both of PVXCP in or into chloroplasts, resulting in a consequential delay in and amelioration of symptom development.

Precursor plastocyanin did not facilitate the entry of PVXCP into isolated pea chloroplasts in co-uptake assays.

We utilized a chloroplast co-uptake experiment to determine whether or not PVXCP could enter isolated chloroplasts with the aid of precursor plastocyanin. In our *in vitro* translation reactions for PVXCP, precursor plastocyanin and mature plastocyanin, the expression level of PVXCP was lower than that of both mature and precursor plastocyanin (Fig. 7, lanes 1 to 3).

The translation products were incubated with intact chloroplasts purified from pea (*Pisum sativum* L) leaves. Our choice

of pea chloroplasts rather than those of *N. benthamiana* was due to the low relative peptide import efficiency of the latter (Y. Qiao and S. M. Wong, *unpublished data*) and the widely cited effective utilization of pea chloroplasts for such assays (Bauerle and Keegstra 1991; Row and Gray 2001). In addition, pea has been shown to be a susceptible host of PVX (Esfandiari et al. 2009). Precursor plastocyanin could enter into chloroplasts and be processed into mature protein (Fig. 7, lane 4), whereas PVXCP and mature plastocyanin could not (Fig. 7, lanes 5 and 6). This suggests that PVXCP cannot enter chloroplasts *in vitro* with the aid of the precursor plastocyanin alone.

DISCUSSION

Previously, we used the CP of *Cymbidium mosaic virus*, a potexvirus, as bait to screen a tobacco cDNA library from which we identified three cDNA clones encoding a precursor plastocyanin (Y. Qiao and S. M. Wong, *unpublished data*). Herein, we have tested the universality of the interaction between plastocyanin and the CP of different potexviruses by investigating PVXCP. Interaction between PVXCP and plasto-

Fig. 5. Subcellular characteristics of chloroplasts isolated from mock-inoculated and *Potato virus X* (PVX)-infected *Nicotiana benthamiana*. **A**, Immuno-gold detection of PVX coat protein (CP) using an anti-PVX antibody and showing immuno-gold particles in the thylakoids (bar = 0.2 μ m). **B**, Negative control, incubated with preimmune serum (bar = 0.2 μ m). **C**, Mock-inoculated leaves (bar = 0.5 μ m) in which the thylakoids appeared in ordered arrays (circled). **D**, PVX-infected leaves (bar = 0.5 μ m) in which disruptions ("loosened" appearance) to the thylakoids were apparent (circled).

cyanin was confirmed both in vitro and in vivo using three independent assays: the yeast two-hybrid system, co-IP, and BiFC analysis. The expression level of plastocyanin was not significantly altered by PVX infection.

From our chloroplast fractionation experiments and TEM observations, we deduced that PVXCP accumulates in chloroplasts (Figs. 4A and 5A) at levels that can be correlated to symptom severity (Fig. 6B and 6C). This is consistent with previous studies that showed that severe chlorotic symptoms of TMV-infected tobacco also correspond to the accumulation of viral CP within the chloroplasts (Lehto et al. 2003; Lindbeck et al. 1991, 1992; Reinero and Beachy 1986). Hefferon and associates (2000) generated PVXCP transgenic potato plants from the chloroplasts of which PVXCP could be isolated, and they suggested that the accumulation of PVXCP within the chloroplasts may play a role in determining viral pathogenicity and symptom development.

In our work, we showed that the thylakoid layers became loosened and the grana disarrayed in the chloroplasts of PVX-infected plants (Fig. 5D). These structural changes are most likely related to changes or disturbances in the thylakoid protein composition and in the functions of the photosynthetic reaction centers or the electron transport chains. These would most likely lead the production of oxygen radicals, oxidative stress, and reduction of chlorophyll. We speculate that this may be the mechanism behind the development of severe mo-

saic symptoms—as has been shown to be the case in infections by *Papaya mosaic virus* (Nanda and Biswal 2008).

Schoelz and Zaitlin (1989) reported that both TMV CP and full-length RNA are present within the chloroplasts of virus-infected leaves, whereas our results showed that PVX RNA was not present in these organelles (Fig. 4B), suggesting that PVXCP is not expressed in chloroplasts but is translocated there. Most chloroplast proteins possess a transit peptide for targeting and entry into the organelle. The CP of *Cucumber necrosis virus*, for example, has a 38-amino acid region that enables such targeting (Xiang et al. 2006), and we asked whether it is possible that PVXCP has a similar transit peptide. Generally, the transit peptides are enriched in hydroxylated amino acid residues and are deficient in acidic residues. They also possess i) an N-terminal region devoid of Gly, Pro, and charged residues, ii) a middle region rich in Ser, Thr, Lys, and Arg, and iii) a C-terminal region predicted to form a β -strand terminating in the stromal processing consensus cleavage site (I/V)X(A/C) $\downarrow$ A (Gavel and von Heijne 1990).

The amino acid sequence of the N-terminus of PVXCP (1 through 80) does not possess any of the characteristic properties that would identify it as a putative transit peptide (Table 1). Furthermore, even if such a domain existed at the N-terminus of PVXCP, it would be cleaved off in the stroma and lumen upon translocation into the chloroplasts, and a diversity of molecular weight CP would, therefore, be detectable during PVX

Fig. 6. Effects of silenced plastocyanin (PC) on viral infection in tobacco. **A**, Relative levels of plastocyanin in *Tobacco rattle virus* (TRV)-infected (T) and plastocyanin-silenced (S) tobacco leaves. Left panels represent semiquantitative reverse transcription-polymerase chain reaction of plastocyanin RNA. Actin RNA was used as a loading control. Right panels represent Western blot analysis of plastocyanin expression in similar tissues. The *RuBisCO* large subunit (RbcL) stained with Coomassie brilliant blue was used to estimate equal loading. Lower panels: Plastocyanin-silenced tobacco showing relatively more severe chlorosis than unsilenced controls. **B**, Plastocyanin-silenced tobacco leaves showing reduced symptom severity following *Potato virus X* (PVX) infection. **C**, The accumulation of PVX coat protein (CP) was reduced in all chloroplast fractions from silenced plants (in comparison with unsilenced controls) at four weeks postinoculation. The expression level of total PVXCP in leaves was not affected.

infections. However, only one CP (at the sequence predicted and previously measured, 28 kDa) could be detected in our purified chloroplasts (Fig. 4A and 6C), providing further indirect evidence that PVXCP does not contain a chloroplast transit peptide.

From the above findings, the presence of PVXCP in the chloroplasts cannot be attributed to an ability of the protein alone nor to translation of the protein within these organelles. Hence, its entry into the chloroplasts is likely to depend upon interactions with other factors.

PVXCP interacted with the transit peptide of plastocyanin (Fig. 1A) and both were collocated to within the chloroplasts (Fig. 2, second row). Knocking down the plastocyanin gene in *N. benthamiana* reduced the accumulation of PVXCP in the chloroplasts but did not completely eliminate it (Fig. 6C). This residual accumulation of PVXCP in the chloroplasts of silenced plants (Fig. 6A, upper left panel—low levels of plastocyanin mRNA could still be detected by RT-PCR in the silenced plants) may have been due to leakiness in gene silencing (Weigel et al. 2003). In addition, as *A. thaliana* has been shown to encode two plastocyanin genes (Gupta et al. 2002), it is possible that additional copies of the plastocyanin gene may also be present in *N. benthamiana* and thereby contribute to the incomplete silencing observed and the consequential translocation of PVXCP into the chloroplasts of silenced plants.

Elaborating on these findings, we conducted pea chloroplast co-uptake assays to clarify whether precursor plastocyanin is the sole factor in determining the entry of PVXCP into chloroplasts. The fluorographic images we obtained indicate that only mature plastocyanin is present in the chloroplasts, that PVXCP was not taken up in these assays, and through the use of precursor plastocyanin as a positive control, that the assay was working effectively.

There are possible explanations for the inability of PVXCP to enter chloroplasts in an in vitro import system that may not completely mimic the normal in vivo conditions. Plastocyanin may only target PVXCP to the surface of the chloroplast, while the entry of the viral protein into the chloroplast may require other molecular chaperones or import mechanisms (Fuks and Schnell 1997; Kessler and Schnell 2006). Alternatively, it

may be that one or more other host proteins (not available in such plastid isolation assays) bring PVXCP to the plastid surface, while the precursor plastocyanin subsequently translocates it into the chloroplast itself.

Such discrepancies between in vivo and in vitro assays have been reported in tobacco (de Boer et al. 1991; de Castro Silva-Filho et al. 1997), *Chlamydomonas reinhardtii* (Kindle and Lawrence 1998; Lawrence and Kindle 1997), and *Arabidopsis* (Rensink et al. 1998). Furthermore, protein accumulation in chloroplasts occurs continuously in vivo, in which the organelle is maintained in its native cellular environment. Therefore, in addition to precursor plastocyanin or one or more other host factors, alone or in combination, the entry of PVXCP into the chloroplasts may require optimal conditions that are only provided in planta.

In totality, our findings suggest that precursor plastocyanin acts as a positive contributor to the localization of PVXCP into chloroplasts and to the consequential symptom phenotype of PVX infection. We speculate that, while possibly not sufficient alone, plastocyanin may play a role either in targeting PVXCP to the surface of the chloroplasts, aid in transporting it inside these organelles by acting as a carrier protein, or both.

Table 1. Sequence analysis of *Potato virus X* coat protein (PVXCP) N-terminal amino acids

Features ^a	Structure (no.) ^b	Remarks
Enriched in hydroxylated residues	Lys (4) Arg (1)	Not enriched
Deficient in acidic residues	Asp (5) Glu (2)	Not deficient
N-terminus without Gly,	Gly (2) Pro (3) Ser (4)	Many charged residues
Pro or charged residues	Glu (1) Thr (9)	
Middle region rich in Ser, Thr, Lys, and Arg	Ser (3) Thr (3) Arg (6)	Uncharacteristic
C-terminal forms a β -strand	Helices and coils	No β -strand predicted
Consensus site (I/V)X(A/C) $\downarrow$ A	Absent	No consensus

^a Consensus features of transit peptides of chloroplast proteins.
^b Structure of the N-terminus and number of transit-peptide consensus N-terminal amino acids in PVXCP.

Fig. 7. Fluorogram of chloroplast co-uptake experiments for plastocyanin and *Potato virus X* coat protein (PVXCP). Lane 1, In vitro translation products of precursor plastocyanin containing the transit peptide, lane 2, precursor plastocyanin with PVXCP, and lane 3, PVXCP with mature plastocyanin lacking the transit peptide region. Lane 4, Proteins extracted from chloroplasts incubated with precursor plastocyanin, lane 5, precursor plastocyanin and PVXCP, and lane 6, mature plastocyanin and PVXCP.

MATERIALS AND METHODS

Plant materials and PVX inoculation.

N. benthamiana plants were grown at 25°C with a 16-h light and 8-h dark photoperiod for four weeks. The plasmid construct p45p46 (Van Wezel et al. 2002) was linearized with *SpeI*, and in vitro transcription was carried out according to the protocol of the mMESSAGEMACHINE kit (Ambion, Austin, TX, U.S.A.). A 1:1 mixture of the resultant transcripts and GKP buffer (50 mM glycine, 30 mM K₂HPO₄, pH 9.2, 1% bentonite, 1% celite) (Petty et al. 1989) was inoculated onto Carborundum-dusted tobacco leaves.

Construction of plasmids.

The full-length coding sequence of the *N. benthamiana* plastocyanin gene was determined by PCR from cDNA using the primers NbPC5F1 and NbPC3R2 (Table 2). NbPC5F1 was a degenerate primer whose design was based upon the plastocyanin gene sequence from *S. lycopersicum* (P17340) and *S. commersonii* (CAJ19272), whereas NbPC3R2 was designed according to the sequence obtained from our library screen. The cDNA of *NbPC* was cloned into pMD18-T (Takara Bio USA, Madison, WI, U.S.A.) and was verified by sequencing.

For constructs used in yeast two-hybrid analyses, the coding sequence of PVXCP was amplified from the full-length PVX cDNA clone p45p46, using the primer pair PVXCPBD5F1 and PVXCPBD3R2. The PCR fragments were restriction digested with *EcoRI/BamHI* and were cloned into the vector pGBKT7 to generate pGBKT7-CP. The full-length coding sequence, transit peptide (residues 1 through 70), and mature protein (residues 71 through 169) of *NbPC* were amplified by PCR, using the primers PCAD5F1 and PCAD3R2, PCAD5F1 and PCTPAD3R2, or PCMPAD5F1 and PCAD3R2, respectively, and were cloned into pGADT7 via the *EcoRI/BamHI* sites to form pGADT7-PC, pGADT7-PCTP, and pGADT7-PCMP, respectively.

For BiFC analysis, the full-length coding sequence of PVXCP was amplified by PCR, using primers PVXCPNE5F1 and PVXCPNE3R2, and was cloned into pUC-SPYNE as a fusion with the N-terminal fragment of YFP via the *BamHI/XhoI* sites, to form pUC-SPYNE-CP. The full-length,

transit peptide (residues 1 through 70) and mature protein (residues 71 through 169) of *NbPC* were PCR-amplified using the respective primer pairs PCCE5F1 and PCCE3R2, PCCE5F1 and PCTPCE3R2, and PCMPCE5F1 and PCCE3R2 and were cloned into pUC-SPYCE as a fusion with the C-terminal fragment of YFP via the *BamHI/XhoI* sites, to form pUC-SPYCE-PC, pUC-SPYCE-PCTP, and pUC-SPYCE-PCMP, respectively (Walter et al. 2004).

To silence plastocyanin gene expression in *N. benthamiana*, a conserved domain consisting of more than 200 nucleotides (residues 292 through 501) was amplified with primers PCTRV5F1 and PCTRV3R2. The amplified product was digested with *BamHI/XhoI* and was inserted into the pTRV2 vector as described previously (Liu et al. 2002), to form the construct pTRV2-PC.

For the in vitro translation experiment, full-length PVXCP, precursor plastocyanin, and mature plastocyanin were amplified with the respective primer pairs: PVXCPT5F1 and PVXCPT3R2, PCPT5F1 and PCPT3R2, or PCMT5F1 and PCMT3R2. The PCR fragments were then cloned into the pGEM-T easy vector (Promega, Madison, WI, U.S.A.) according to the manufacturer's instructions.

Yeast two-hybrid and co-immunoprecipitation assays.

Yeast two-hybrid assays were carried out using the Matchmaker GAL4 two-hybrid system. *Saccharomyces cerevisiae* AH109 was transformed with the yeast two-hybrid constructs described above, using the small-scale lithium acetate method, and was subsequently plated on SD minimal medium lacking adenine, histidine, leucine, and tryptophan (SD/-Ade/-His/-Leu/-Trp) according to the BD Matchmaker library construction and screening kit user manual (BD Biosciences, Rutherford, NJ, U.S.A.).

In vivo co-immunoprecipitation was done in accordance with the manufacturer's protocol (Pierce, Rockford, IL, U.S.A.). Briefly, PVX-infected leaves (0.1 g) were homogenized on ice in 0.2 ml of protein extraction buffer (220 mM Tris-HCl, pH 7.4, 250 mM sucrose, 50 mM KCl, 1 mM MgCl₂, 2 mM phenylmethylsulfonyl fluoride, 10 mM β-mercaptoethanol, and 1× complete EDTA-free protease inhibitor (Sigma, St. Louis). The extract was centrifuged at 12,000 × g for 10 min to

Table 2. Primers used in plasmid construction

Primers	Primer sequences	Restriction sites	Constructions
NbPC5F1	5'-ATGGCCACHGTYRCYTC-3'	...	pMD18T-PC
NbPC3R2	5'-TTAGTTAACAGTAACTTTGCC-3'	...	pMD18T-PC
PVXCPBD5F1	5'-CGGAATTCATGTCAGCACCAGCTAGCAC-3'	<i>EcoRI</i>	pGBKT7-CP
PVXCPBD3R2	5'-CGGGATCCCTTATGGTGGTGGTAGAGTG-3'	<i>BamHI</i>	pGBKT7-CP
PCAD5F1	5'-CGGAATTCATGGCCACCGCTACCTCTGC-3'	<i>EcoRI</i>	pGADT7-PC
PCAD3R2	5'-CGGGATCCCTTAGTTAACAGTAACTTTGC-3'	<i>BamHI</i>	pGADT7-PC
PCTPAD3R2	5'-CGGGATCCAGCCATGGCATTGCTAGCAAG-3'	<i>BamHI</i>	pGADT7-PCTP
PCMPAD5F1	5'-CGGAATTCATTGAAGTGTTGCTTGGTAG-3'	<i>EcoRI</i>	pGADT7-PCMP
PVXCPNE5F1	5'-CGGGATCCATGTCAGCACCAGCTAGCAC-3'	<i>BamHI</i>	pUC-SPYNE-CP
PVXCPNE3R2	5'-CCGCTCGAGTGGTGGTGGTAGAGTG-3'	<i>XhoI</i>	pUC-SPYNE-CP
PCCE5F1	5'-CGGGATCCATGGCCACCGTCACCTCTGC-3'	<i>BamHI</i>	pUC-SPYCE-PC
PCCE3R2	5'-CCGCTCGAGGTTAACAGTAACTTTGCC-3'	<i>XhoI</i>	pUC-SPYCE-PC
PCTPCE3R2	5'-CCGCTCGAGATGGCATGGCTGCTAGCAAG-3'	<i>XhoI</i>	pUC-SPYCE-PCTP
PCMPCE5F1	5'-CGGGATCCATGATTGAAGTGTGCTTGG-3'	<i>BamHI</i>	pUC-SPYCE-PCMP
FLPC5F1	5'-GGCTATTGAAGTGTTGCTTGGT-3'	...	...
FLPC3R2	5'-GCTTACGCTGAAGTTTCCAGG-3'	...	...
PCTRV5F1	5'-CGGGATCCACATTCACAGAACAATGC-3'	<i>BamHI</i>	pTRV2-PC
PCTRV3R2	5'-CCGCTCGAGAGTAACTTTGCCAACC-3'	<i>XhoI</i>	pTRV2-PC
PVXCPT5F1	5'-CGGGATCCATGTCAGCACCAGCTAG-3'	...	pT/T-PVXCP
PVXCPT3R2	5'-CGAGCTCTTATGGTGGTGGTAGAGTGAC-3'	...	pT/T-PVXCP
PCPT5F1	5'-ATGGCCACCGTCACCTCTGCTGC-3'	...	pT/T-PCP
PCPT3R2	5'-TTAGTTAACAGTAACTTTGCCAACC-3'	...	pT/T-PCP
PCMT5F1	5'-ATGATTGAAGTGTGCTTGGTAGCG-3'	...	pT/T-PCM
PCMT3R2	5'-TTAGTTAACAGTAACTTTGCCAACC-3'	...	pT/T-PCM

^a ... indicates no restriction sites were added.

remove cellular debris, and the supernatant (100- μ l aliquots) was mixed with equal volumes of coupling buffer and incubated for 2 h with amine-reactive gel previously coupled to 50 μ l of rabbit anti-plastocyanin antibody or preimmune serum. The amine-reactive gel was washed three times with coupling buffer and the immunoprecipitated proteins were subsequently eluted from the gel with elution buffer. The immunoprecipitates were probed with anti-PVX and anti-plastocyanin antibodies.

Isolation of protoplasts.

Fully expanded leaves were collected from four- to five-week-old *N. benthamiana* seedlings and were surface sterilized for 5 min with 0.8 % Clorox (sodium hypochlorite), followed by rinsing with autoclaved Milli-RO water (three times for 5 min each). The leaves were sliced into 1-mm strips and were incubated in an enzyme mixture (0.5 M mannitol, 22 mM KNO₃, 0.5 mM morpholineethanesulfonic acid [MES], 1 μ M KI, 10 mM CaCl₂, 0.1 μ M CuSO₄, 0.75 mM KH₂PO₄, 0.5% cellulase R10, and 1% macerozyme R10 [Yakult Honsha, Tokyo], pH 5.7) overnight at 25°C in the dark. Protoplasts were released and passed through a 70- μ m cell strainer before rinsing with wash buffer (10 mM HEPES [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid], 150 mM NaCl, 0.5 M mannitol, 10 mM CaCl₂, pH 5.7). Prepared protoplasts (2×10^5) were counted for transfection with 10 μ g of the relevant plasmid (slowly added into a mixture of 100 μ l of polyethylene glycol [PEG] solution [40% PEG, 10 mM CaCl₂] and 4.5 ml of wash buffer). The protoplast solution was mixed well and kept on ice for 30 min before centrifugation at $40 \times g$ for 7 min. The supernatant was discarded and the protoplasts washed again with 5 ml of wash buffer to remove residual PEG. Protoplasts were incubated in petri dishes with 3 ml of protoplast culture medium (Murashige-Skoog medium containing 0.5 M mannitol and 10 mM CaCl₂) at 25°C in the dark.

BiFC assay and confocal laser scanning microscopy.

The vectors used in the BiFC assay were provided by J. Kudla from the Universität Münster, Münster, Germany. Transient transfections of tobacco protoplast cultures with combinations of pUC-SPYNE-CP/pUC-SPYCE-PC, pUC-SPYNE-CP/pUC-SPYCE-PCTP, pUC-SPYNE-CP/pUC-SPYCE-PCMP, and pUC-SPYNE/pUC-SPYCE were performed as described previously (Walter et al. 2004). YFP fluorescence could be detected in tobacco protoplasts 22 to 36 h after transfection. Confocal microscopy was performed on an inverted spectral confocal laser scanning microscope (Carl Zeiss LSM 510, Munich). Samples were excited using a 514 nm argon laser with an emission band of 530 to 600 nm for YFP detection or 650 nm to detect chlorophyll autofluorescence from the *N. benthamiana* chloroplasts.

RNA extraction and real time RT-PCR analysis.

Total RNA was extracted from the plant tissues using TRIzol (Invitrogen, Carlsbad, CA, U.S.A.). The RNA was treated with 2 U of RNase-free DNase (Promega) at 37°C for 1 h and was recovered by ethanol precipitation. Total RNA extracted from chloroplasts isolated from mock-inoculated or PVX-infected leaves was carried out as described above with the addition of an RNase preincubation step on the isolated chloroplasts (1 h on ice) prior to extraction (Schoelz and Zaitlin 1989). This additional step allowed for the degradation of any potentially contaminating RNA present on the outer surface of the chloroplast envelope.

Real-time RT-PCR assays were performed using the plastocyanin-specific primers FLPC5F1 and FLPC3R2 (Table 2). Briefly, first-strand cDNA was synthesized from 1 μ g of total RNA, using AMV reverse transcriptase (Promega). A typical

reaction consisted of 100 ng of the reverse transcribed cDNA, 200 nM each of the sense and antisense gene-specific primers, and 5 μ l of SYBR green master mix, in a total volume of 10 μ l and in accordance with the manufacturer's instructions (Applied Biosystems, Foster City, U.S.A.). The PCR conditions were 10 min at 95°C, followed by 40 cycles of 15 s at 95°C, 30 s at 60°C, and 45 s at 72°C.

Real-time RT-PCR quantification was based upon relative abundance, as determined by plastocyanin Ct values (Ct being the threshold cycle value) compared with actin Ct values according to the formula $\Delta Ct = 2^{-(Ct_{pc} - Ct_{actin})}$.

Purification and fractionation of chloroplasts.

Chloroplasts were purified from healthy and PVX-infected tobacco leaves through sucrose density gradient centrifugation as described previously (Cseke et al. 2004). Tobacco plants were kept in the dark for 12 h to reduce starch content before harvesting young leaves. The leaves (5 g) were rinsed with 5% Clorox for 4 min and were then rinsed with cold sterile milli-RO water. The surface-sterilized leaves were cut into 1- to 2-mm-wide strips and were transferred into a blender containing 30 ml of isolation buffer (350 mM sorbitol, 50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 0.1% bovine serum albumin, 0.1% β -mercaptoethanol, and 0.1% sodium ascorbate). The homogenate was filtered through five layers of cheesecloth and was centrifuged at $3,000 \times g$ for 15 min. The pellet was resuspended with 2 ml of washing buffer (350 mM sorbitol, 50 mM Tris-HCl, pH 8.0, and 1 mM EDTA), and the crude preparation of chloroplasts was layered over a 30:45:60% sucrose gradient for centrifugation at $80,000 \times g$ (using a Beckman SW28 rotor) for 1 h at 4°C. A single dark-green band of chloroplasts at the interface between the 30 and 45% sucrose gradient steps was removed with a Pasteur pipette. Four volumes of washing buffer were added, and the chloroplast suspension was recentrifuged at $6,000 \times g$ for 10 min to remove any remaining sucrose. The pellet was finally resuspended in 2 ml of washing buffer before proteins associated with the outer surface of the envelope were removed by incubating the purified chloroplasts with thermolysin at 200 μ g/ml (Sigma) on ice for 30 min. Chloroplasts were subsequently washed with washing buffer containing 5 mM EDTA. The isolated intact chloroplasts were counted under the light microscope using a hemocytometer. From the 1-ml chloroplast suspension, 5 μ l was withdrawn and diluted to 100 μ l and was quantified accordingly. The final count for each test sample was adjusted to 1×10^8 chloroplasts by buffer dilution.

The chloroplast pellet was prepared for fractionation by resuspension in lysis buffer (10 mM HEPES, pH 8.0, and 5 mM MgCl₂). Following centrifugation at $20,000 \times g$ for 10 min, the supernatant contained the stroma and envelope fractions and the pellet the intact thylakoid fraction. The supernatant was centrifuged again ($140,000 \times g$ for 10 min) to separate stroma (supernatant) and envelope (pellet). The pellet from the initial centrifugation step (containing intact thylakoids) was subjected to five cycles of freezing and thawing, using liquid nitrogen, to allow the thylakoid membranes to be separated from the lumen by centrifugation ($12,000 \times g$ for 10 min).

Polyclonal antibody production and Western blots.

The recombinant clone pET-PC in *Escherichia coli* BL21 cells was used for antibody production as follows. The full-length coding sequence of plastocyanin was cloned in frame into the pET28a vector. The resultant recombinant protein expressed by this construct was overproduced in *E. coli* and was purified by excising the relevant SDS-PAGE gel band. Purified plastocyanin was resuspended in TBS buffer (50 mM Tris HCl, 150 mM NaCl, pH 7.4) and was used to inject a rabbit for anti-serum production.

Protein levels loaded onto denaturing SDS-PAGE gels were equalized by determining total protein levels via direct visualization with Coomassie blue staining. Estimated equivalent amounts of each sample were again separated by SDS-PAGE for blotting onto nitrocellulose membranes. Blotted proteins were probed with anti-PVX or anti-plastocyanin antibody followed by alkaline phosphatase-conjugated protein A and were visualized using nitroblue tetrazolium/5-bromo-4-chloroindol-3-yl phosphate staining.

Silencing plastocyanin by VIGS.

A VIGS construct containing a fragment of the plastocyanin gene from *N. benthamiana* was made using a TRV vector to produce pTRV2-PC. As a nonsilenced control, *N. benthamiana* plants were infected with TRV alone. Constructs pTRV1, pTRV2 (Liu et al. 2002), and pTRV2-PC were introduced into *Agrobacterium tumefaciens* GV3101 by heat shock. Discrete 2-ml cultures of GV3101 containing the constructs pTRV2, pTRV2-PC, empty vector control, or pTRV1 were grown overnight at 28°C in Luria-Bertani medium containing 50 µg of kanamycin per milliliter and 12.5 µg of rifampicin per milliliter. Overnight cultures were pelleted, were resuspended in infiltration buffer (10 mM MgCl₂, 10 mM MES, and 100 µM acetosyringone), and were adjusted to an optical density at 600 nm value of 0.8. The pTRV1 *Agrobacterium* culture was then mixed with an equal volume of the pTRV2 or pTRV2-PC cultures. The mixtures were incubated at room temperature for 3 h before infiltration onto the abaxial side of leaves from four-week-old *N. benthamiana* seedlings using a 3-ml needleless syringe (agroinfiltration). Seedlings were maintained for two weeks at 24°C in growth chambers before the top, fully expanded leaves were removed to check the plastocyanin silencing efficiency (by Western blot of total protein extracts). Plants in which plastocyanin was confirmed to be silenced by Western analysis were then inoculated with the p45p46 in vitro transcript. After 30 days, chloroplasts were isolated from the top leaves (showing severe mosaic symptoms of PVX infection) for subsequent analysis of PVXCP accumulation in the different chloroplast fractions.

In vitro translation and co-uptake assays.

The PVX CP, precursor plastocyanin and mature plastocyanin were synthesized in the presence of (35S)-labeled methionine (Met) (Perkin-Elmer, Norwalk, CT, U.S.A.) by coupled in vitro transcription or translation of respective cDNA clones in a wheat germ system (Promega). The co-uptake assay essentially followed that of Rensink and associates (1998). The isolated pea chloroplasts were purified using Percoll gradient centrifugation. The pelleted chloroplasts were resuspended in ice-cold import buffer (330 mM sorbitol, 50 mM HEPES/KOH, pH 8.0). For total chlorophyll content determination, the method described by Arnon (1949) was followed. In brief, 10 µl of chloroplast suspension was diluted to 1 ml of 80% acetone solution and mixed. After centrifugation for 2 min at 3,000 × g, supernatant was retained and the absorbance was measured at 652 nm. The amount of chlorophyll per milliliter was obtained by multiplying the absorbance by the dilution factor and dividing by the extinction coefficient of 36. To each sample containing chloroplasts with 50-µg equivalent of chlorophyll, 1.5 mM dithiothreitol and 10 mM magnesium-ATP were added to make a final volume of 100 µl. The samples were incubated in the dark at 25°C for 10 min. Translation mixture (50 µl) and 5 mM unlabeled Met in import buffer were added, and the samples reincubated at 25°C in the light for 30 min, before sedimenting by centrifugation and resuspension of the pellet in import buffer. The chloroplasts were

finally treated with thermolysin (200 µg/ml) in 1 mM CaCl₂ and were repurified on 40% Percoll. Plastid proteins were analyzed by SDS-PAGE gel and fluorography.

TEM.

Both healthy and PVX-infected *N. benthamiana* leaves were fixed for 2.5 h at 4°C in 0.1 M sodium phosphate buffer (pH 7.2) containing 2.5% glutaraldehyde and 4% paraformaldehyde (Morales et al. 2001). Tissues were then postfixed with 1% osmium tetroxide for 2 h before dehydration through a graded alcohol series and embedding in Spurr's resin. Blocks were sectioned on a Reichert ultramicrotome. Thin sections for TEM were placed on copper grids and were stained with 2% (wt/vol) aqueous uranyl acetate, followed by 10% (wt/vol) lead citrate. Immuno-EM was performed essentially as described previously (Suzuki and Kataoka 1992; Tomoyasu et al. 1993), except that the leaves were cut into 1 × 1-mm pieces and were fixed with 0.1% glutaraldehyde and 4% paraformaldehyde in sodium cacodylate (pH 7.4) under vacuum. The tissues were embedded in LR white resin (London Resin Co., Reading, U.K.) and ultrathin sections were cut on an ultramicrotome, before incubation with the anti-PVX antibody (diluted 1:250). Washed sections were incubated with anti-rabbit IgG conjugated with 15-nm diameter gold particles (diluted 1:100; Biocell Research Laboratories, Cardiff, U.K.) for 30 min at 37°C. After immunolabeling, sections were stained with uranyl acetate and lead citrate before observation of the ultrastructures using a Philip CM-10 transmission electron microscope (Eindhoven, The Netherlands). As the cytochemical control, specimens were incubated with preimmune serum.

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