Identification of a *Plum pox virus* CI-Interacting Protein from Chloroplast That Has a Negative Effect in Virus Infection

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Submitted 23 September 2005. Accepted 18 November 2005.

The cylindrical inclusion (CI) protein of potyviruses is involved in virus replication and cell-to-cell movement. These two processes should rely on multiple plant–virus interactions; however, little is known about the host factors that are involved in, or that may interfere with, CI functions. By using a yeast two-hybrid system, the CI protein from *Plum pox virus* (PPV) was found to interact with the photosystem I PSI-K protein, the product of the gene *psaK*, of *Nicotiana benthamiana*. Coexpression of PPV CI was shown to cause a decrease in the accumulation level of PSI-K transiently expressed in *N. benthamiana* leaves. To test the biological relevance of this interaction, we have analyzed the infection of PPV in *N. benthamiana* plants in which *psaK* gene expression has been silenced by RNA interference, as well as in *Arabidopsis thaliana* *psaK* knockout plants. Our results show that downregulation of the *psaK* gene leads to higher PPV accumulation, suggesting a role for the CI–PSI-K interaction in PPV infection.

Additional keywords: host proteins, RNA helicase, RNA silencing, sharka disease, viral proteins.

Host factors play an essential role in virus infection. In the case of plant viruses, infection depends on a number of processes such as viral genome amplification, cell-to-cell movement, and long-distance movement, in which multiple interactions between viral and host factors are taking place. Defense mechanisms such as the hypersensitivity response or posttranscriptional gene silencing and their suppression by viral proteins also involve interactions between the plant and the pathogen. These interactions may cause symptom development, although symptoms also can be a consequence of other collateral interactions that are irrelevant to the virus replication. Thus, identification of interactions between viral and host proteins is of growing interest, and there are many examples of host factors whose role in virus infection has been characterized (Whitham and Wang 2004).

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The nucleotide sequence data from *N. benthamiana* *psaK* mRNA is available in the GenBank nucleotide sequence database under the accession number AY899937.

*The e-Xtra logo stands for “electronic extra” and indicates that Figures 3 and 5 appear in color online.

Plum pox virus (PPV) is a potyvirus that causes sharka disease in fruit trees of the *Prunus* genus and also can infect different herbaceous hosts (López-Moya et al. 2000). The potyviral genome consists of a positive polarity single-stranded RNA molecule and codes for a single polyprotein that is processed by three virus-encoded proteases (Revers et al. 1999; Riechmann et al. 1992). A unique feature of the potyvirus infection is the accumulation of characteristic pinwheel-shaped cytoplasmic inclusion bodies that are formed by the cylindrical inclusion (CI) protein. The potyviral CI protein has NTPase and RNA helicase activities (Eagles et al. 1994; Laín et al. 1990, 1991), which have been shown to be required for RNA replication (Fernández et al. 1997). Electron microscopy analysis of CI structures associated to plasmodesmata (Roberts et al. 1998; Rodríguez-Cerezo et al. 1997) and mutational studies of the CI protein from *Tobacco etch virus* (TEV) (Carrington et al. 1998) have indicated that the CI protein also is involved in cell-to-cell movement of potyviruses.

The role of the CI protein in both virus replication and cell-to-cell movement, as well as its relevance in the activity of at least one resistance gene (Jenner et al. 2000, 2002), suggest that different host factors may interact with the CI protein during virus infection. Although an interaction between the helicase domain of the CI protein from TEV and the protein P58^{IPK} from tobacco has been reported (Bilgin et al. 2003), little is known about the host proteins that interact with the potyviral CI protein and their role in virus infection. The yeast two-hybrid system (Fields and Song 1989) previously has been used to identify host proteins that interact with potyviral proteins such as HCPro (Anandalakshmi et al. 2000; Guo et al. 2003), VPg (Dunoyer et al. 2004; Leonard et al. 2000; Schaad et al. 2000; Wittmann et al. 1997), or Nib (Wang et al. 2000). In this study, the PPV CI protein has been used as a bait to screen a *Nicotiana benthamiana* cDNA library by the two-hybrid system. The PSI-K subunit of photosystem I has been identified as a CI-interacting protein. The relevance of this interaction has been studied by analyzing the effect of interfering with PSI-K expression in PPV infection.

RESULTS

Library screenings with the PPV CI protein bait.

The activities of the CI protein in replication and movement, two important processes for virus infection, are expected to involve multiple interactions with different host factors. Thus, the yeast two-hybrid system was used to identify plant CI-interacting proteins in a cDNA library from *N. benthamiana*. In a first screening, using pLex9-CI as bait, two clones were found to

code for CI-interacting products. Ten clones were identified in a second screening using pAS-CI plasmid as bait, which included the two clones selected in the first screening. However, using a more selective yeast strain (PJ69/4a), only the two clones identified in both screenings allowed yeast growth in minimal medium. These two clones were selected for further analysis.

Sequence analysis showed that one of the clones coded for a 31-amino-acid (aa) peptide, which corresponded to an unknown protein. The second clone coded for a 130-aa protein that was highly similar to the photosystem I PSI-K protein of several plants, with identity levels of 50 to 85%; the clone also included 5' and 3' noncoding regions of 33 and 48 nucleotides

(nt), respectively, and was named pACT-PSIK. We used a rapid amplification of cDNA ends (RACE) method to obtain the *psaK* mRNA complete sequence. The RACE product was identical to the library clone. PSI-K is encoded by a nuclear gene (*psaK*) and is synthesized as a precursor in the cytoplasm and transported to the chloroplast, where it is processed and localized in the nonappressed region of the thylakoid membranes (Chitnis 1996).

We analyzed in more detail the CI-PSI-K interaction in the two-hybrid system, using the PJ69/4a yeast strain (Fig. 1). Positive interactions of PSI-K with an N-terminal CI fragment of 409 aa (CI409) were detected. The CI409 fragment includes the

Fig. 1. Interactions detected in the two-hybrid system. **A**, Schematic representation of *Plum pox virus* genome and the cylindrical inclusion (CI) fragments used in this study. The seven conserved motifs of RNA helicases are represented as black boxes. **B**, Ability to grow in minimal medium containing adenine and histidine (+A+H), adenine (+A-H), or lacking adenine and histidine (-A-H) of yeast cells containing the indicated plasmids. β -Galactosidase levels (picograms per microgram of total protein, means of at least six independent colonies $\pm$ standard deviation) are shown for most of the tests. BG, background activity, corresponding to less than 10 pg/ μ g of total protein. ND means that β -galactosidase activity was not determined.

seven conserved motifs of the RNA helicases and has been shown to self-interact in the two-hybrid system (López et al. 2001). PSI-K did not interact with other CI fragments, including the CI177 fragment, which also is involved in CI–CI interactions (López et al. 2001). Positive interactions were not detected between PSI-K and any other PPV protein analyzed, such as coat protein (CP), NIb, or P3/6k1 (data not shown). The interaction between the two-hybrid proteins induced activation of the β -galactosidase (β -gal) reporter gene. Quantitative analysis of the resulting β -gal activity was used to more accurately assess the strength of the interaction. β -Gal levels in yeast transformed with pACT-PSIK and pAS-CI or pAS-CI409 were comparable with those detected in yeast transformed with the control plasmids pVA3-1 and pTD1-1, indicating that CI-PSI-K and CI409-PSI-K interacted with high efficiency (Fig. 1).

To test whether these interactions were specific for PPV in *N. benthamiana* or might represent a more general event in potyvirus infection, we analyzed the possible interactions of PPV CI with the PSI-K protein from *Arabidopsis thaliana* (atPSI-K, locus At1g30380) and the interactions of the CI protein of a different potyvirus, *Tobacco vein-mottling virus* (TVMV), with the PSI-K protein of these two plant species. Yeasts transformed with pAS-CI and pACT-atPSIK were not able to grow in the absence of histidine and adenine, but grew in less restrictive conditions, when the minimal medium was supplemented with adenine (Fig. 1), suggesting that PPV CI interacts with AtPSI-K, although the interaction could be weaker than that with *N. benthamiana* PSI-K. This result was supported by the values of β -gal activity of the yeasts harboring pAS-CI and pACT-AtPSIK, which clearly were above background levels but were lower than those of yeasts harboring pAS-CI and pACT-PSIK (Fig. 1). AtPSI-K also interacted with the PPV CI409 fragment. Curiously, although this interaction allowed transformed cells to grow only in low-stringency restrictive conditions, it gave rise to high β -gal levels, similar to those of yeasts transformed with the positive control plasmids (Fig. 1).

TVMV CI also was able to interact with PSI-K from both plant species, allowing the transformed yeasts to grow in the absence of histidine, although not in the absence of adenine (Fig. 1). The levels of β -gal activity of yeasts transformed with pAS-CITVMV and pACT-PSIK were low and those of yeasts transformed with pAS-CITVMV and pACT-PSIK were not

significantly above background, suggesting that the interactions with PSI-K of TVMV CI are weaker than those of PPV CI.

Virus infection affects the expression level of the PSI-K-encoding mRNA.

In contrast with previous results showing that barley *psaK* mRNA levels increased upon illumination (Kjaerulff et al. 1993), we did not observe differences in expression of the *N. benthamiana* *psaK* gene between leaves harvested after 9 h in the dark and after 2 or 8 h of illumination (Fig. 2A). Similar levels of *psaK* mRNA were detected on upper distal leaves from either PPV-infected or mock-treated plants (Fig. 2B). However, PPV infection appeared to result in a decrease in *psaK* mRNA accumulation in the inoculated leaves (Fig. 2B).

Silencing of the *psaK* gene facilitates PPV infection in *N. benthamiana*.

To assess the possible role of the CI–PSI-K interaction during PPV infection, RNAi technology was used to inhibit *psaK* gene expression in *N. benthamiana*, and PPV infection was tested in the *psaK*-silenced plants. Plasmid pdsPSIK, which contains two copies of a 320-nt fragment from the *psaK* gene placed in opposite orientation and separated by an intron, was constructed (Fig. 3A). Transient silencing assays were carried out by infiltration of *N. benthamiana* leaves with a suspension of *Agrobacterium tumefaciens* (strain C58C1) that carried pdsPSIK. After 4 days, the agroinfiltrated leaves were inoculated mechanically with a recombinant PPV that expresses the green fluorescent protein (GFP), so that we could easily follow the virus spread. The number of GFP foci monitored at 5 days post inoculation (dpi) was higher in the leaves infiltrated with an *Agrobacterium* sp. carrying pdsPSIK than in the control plants (15.8 ± 4.0 and 7.7 ± 1.3 per leaf, respectively, as an average of three independent experiments). Northern blot analysis from these leaves confirmed the silencing of the *psaK* gene, suggesting that the differences in the GFP-foci number were due to the absence of the PSI-K protein (data not shown).

The higher infection rate obtained in the transiently silenced plants prompted us to obtain *N. benthamiana* transgenic plants, in which the *psaK* gene is constitutively silenced, by stable transformation with the pdsPSIK construct. Efficient silencing of the *psaK* gene was shown by Northern blot analysis in five of the nine transgenic lines in which the dsPSIK was detected by polymerase chain reaction (PCR) (Fig. 3B; data not shown). Three lines with single insertion loci were chosen for the further analysis of susceptibility to PPV: dsPSIK-5, in which the *psaK* expression is silenced; dsPSIK-6, not silenced; and the vector-transformed line S3. These lines were phenotypically indistinguishable.

Infection rate of PPV GFP in the transgenic plants was evaluated, and the results shown in Figures 3C and 4 are representative of those obtained in two independent experiments. The number of GFP expression foci was slightly higher in the inoculated leaves of dsPSIK-5 plants than in those of dsPSIK-6 or S3 plants, and more intense GFP fluorescence was observed in both inoculated and distal infected leaves of plants of the dsPSIK-5 line compared with those of the control plants (Fig. 3). Enzyme-linked immunosorbent assay (ELISA) data showed that PPV accumulation was higher in plants from the dsPSIK-5 line than in the dsPSIK-6 and S3 control plants (Fig. 4), mainly at early times of infection (5 dpi for the inoculated leaves and 10 dpi for the upper distal leaves). The attenuation of the effect of the dsPSIK-5 transgene later on PPV infection was not a consequence of reactivation of *psaK* gene expression by the silencing suppression activity of HCPro encoded by PPV (Sáenz et al. 2002), because Northern blot analysis showed that *psaK* silencing was maintained even at 21 dpi (data not shown).

Fig. 2. Expression levels of the *Nicotiana benthamiana* *psaK* gene. **A**, Northern blot analysis of *psaK* mRNA levels along the day. Samples were harvested at night (lane 1) and at 2 h (lane 2) and 8 h (lane 3) after illumination. **B**, Northern blot analysis of *psaK* gene expression in inoculated (I) or systemic (S) leaves of mock-inoculated and *Plum pox virus* (PPV)-infected plants. Each sample corresponds to a pool of three independent plants.

PPV infection on PSI-K knockout *Arabidopsis* plants.

Silencing of the *psaK* gene by RNAi in *N. benthamiana* was a useful approach to analyze the role of the PSI-K protein in PPV infection. However, RNA silencing usually does not completely abolish gene expression and we cannot discard the possibility that residual expression of the *psaK* gene in the dsPSIK-5 plants could lead to enough protein accumulation to ensure some level of activity. For this reason, we searched in the literature for PSI-K knockout mutants, in which the PSI-K protein could be totally absent. Several *Arabidopsis* knockout mutants of photosystem I proteins have been described (Varotto et al. 2002). These authors kindly supplied us the *psak-1* and *psag-1.4* mutants, which lack PSI-K and PSI-G, another photosystem I protein, respectively, which is evolutionarily related to PSI-K (Kjaerulff et al. 1993). Neither of these two mutants displayed apparent phenotypic defects.

Rosette leaves of mutants and wild-type (Col-0) plants were inoculated with PPV GFP by agroinfiltration and the pattern of GFP expression was monitored. The number of GFP expression foci and their rate of spread were higher in the inoculated leaves of the *psak-1* mutant than in those of the *psag-1.4* mutant or the wild-type plants (Fig. 5A). The higher GFP expression in the *psak-1* mutant correlated with a higher PPV accumulation, as measured by ELISA, than in the control plants (Fig. 5B). These results were reproduced in the inoculated leaves of three independent experiments. However, when systemically infected

leaves were analyzed (both rosette and caulinar leaves), the pattern of PPV accumulation was irregular, with high variations among plants and experiments, preventing us from drawing confident conclusions about the effect of the *psak* mutation on the infection of systemic tissues (data not shown).

Coexpression of PPV CI has a negative effect on the accumulation level of transiently expressed PSI-K.

To further analyze the interaction of CI with the PSI-K protein in planta, we aimed to express tagged versions of the two proteins in *N. benthamiana*. The PPV CI protein was tandem affinity purification (TAP) tagged (TAP-CI) at its N-terminal end, whereas a C-terminal hemagglutinin epitope (HA) tag was used for PSI-K (PSIK-HA). Leaves of young *N. benthamiana* plants were agroinfiltrated with the appropriate constructs and the expression of the fusion proteins was analyzed by using specific antibodies against any of the tags. In plants agroinfiltrated with a single construct, the expression of both PSIK-HA and TAP-CI proteins was detected at 3, 6, and 9 days post agroinfiltration (Fig. 6; results not shown). For PSIK-HA, two bands could be detected, probably corresponding to the precursor and the mature protein forms (Fig. 6A). However, coagroinfiltration of leaves with both constructs to express PSIK-HA and TAP-CI simultaneously led to a strong reduction in the expression level of PSIK-HA, which was noticeable at all times post agroinfiltration analyzed (3, 6, and 9 days) (Fig. 6A; results not shown).

Fig. 3. Plum pox virus (PPV) infection of *Nicotiana benthamiana* transgenic plants transformed with a *psaK*-derived inverted repeat transgene. **A**, Schematic representation of *psaK* mRNA and the *pdsPSIK* inverted repeat construct. The region of the *psaK* mRNA used in the construction and its size are indicated. The white arrow represents the *Cauliflower mosaic virus* 35S promoter, whereas the intron and the nopaline synthase terminator are represented as a white and a black rectangle, respectively. **B**, Northern blot showing *psaK* gene expression in transgenic plants transformed with *pdsPSIK* (lines 5 and 6). Line S3 is a vector-transformed plant. **C**, Green fluorescent protein (GFP) expression foci in inoculated leaves at 5 and 10 days postinoculation (dpi) and systemically infected leaves at 10 dpi of transgenic plants infected with PPV-GFP. Bar = 1 cm.

The reduced accumulation of PSIK-HA protein in the presence of TAP-CI was not an unspecific effect of the coagroinfiltration system, because the coexpression of TAP-CI together with GFP did not affect the accumulation level of GFP (Fig. 6B). In addition, when PSIK-HA was coexpressed with HCPro from PPV, also TAP-tagged at its N-terminal end, high levels of PSIK-HA were obtained (Fig. 6A), suggesting that the strong reduction in the accumulation level of PSIK-HA takes place only when TAP-CI and PSIK-HA proteins are expressed together; we have no obvious explanation for the change in relative intensity of the two PSIK-HA bands associated with TAP-HCPro coexpression (Fig. 6A). Northern blot analysis showed no differences in PSIK-HA or GFP mRNA levels associated with coagroinfiltration with the TAP-CI-expressing plasmid, demonstrating that TAP-CI was not affecting synthesis efficiency or stability of the PSIK-HA mRNA (data not shown).

DISCUSSION

Information about host proteins that interact with viral proteins is of key importance to characterize the molecular basis of the infection process and the development of antiviral strategies. In this work, we have identified the photosystem I PSI-K subunit as a protein that interacts with the potyvirus CI protein in the yeast two-hybrid system, and we present data suggesting that this interaction has a role in PPV infection.

The two-hybrid assays performed in this study demonstrated the specificity of the CI-PSI-K interaction, because no interactions were detectable between PSI-K and other PPV proteins such as Nib, P3/6k1, or CP. The interaction first was detected between CI from PPV and PSI-K of *N. benthamiana*; however, we also demonstrated that the CI of another potyvirus (TVMV)

and the PSI-K protein of another plant species (*Arabidopsis*) could be partners in the interaction. Interactions played by TVMV CI or *Arabidopsis* PSI-K were detected only when the selection media was supplemented with adenine, suggesting that they represent weak interactions, although they also were very specific. The binding strength in the two-hybrid system does not necessarily correlate with the affinity level under natural conditions because, among other reasons, subtle differences in the expression levels or stability of the hybrid proteins, which have not been thoroughly approached in this study, can affect the apparent intensity of the interaction; therefore, we cannot predict how the quantitative differences that we have observed in the yeast experiments could contribute to differences in the efficiency of the virus infection. Because both PPV and TVMV systemically infect *N. benthamiana* and *Arabidopsis* plants, it could be interesting to select noncompatible potyvirus-plant combinations to assess the possible contribution of CI-PSI-K interactions to host range determination. The fact that the CI protein from two different potyviruses interacts with the PSI-K protein from two different plant species suggests that the CI-PSI-K interaction could have a functional relevance in plant infection by potyviruses.

The use of different PPV CI segments showed that regions involved in the CI-PSI-K interaction must be present along the CI409 fragment, which includes the seven conserved motifs of RNA helicases, but did not allow us to define a precise interaction domain in the CI protein.

Posttranscriptional gene silencing is a powerful tool to obtain loss of function mutants suitable to investigate the role of a given gene. This approach has been used previously to study the function of the *psaK* gene from *Arabidopsis* (Jensen et al. 2000), showing that PSI-K is involved in organizing the peripheral light-harvesting complexes on the core antenna of photo-

Fig. 4. Virus accumulation measured by double-antibody sandwich indirect enzyme-linked immunosorbent assay in *Plum pox virus*-infected *Nicotiana benthamiana* transgenic plants from lines S3 (vector-transformed), dsPSIK-5 (*psak* silenced), and dsPSIK-6 (not silenced). Values are the mean of three **A**, inoculated or **B**, systemically infected leaves from four plants per line, and every sample was assayed in duplicate. Standard deviation values are shown; dpi = days postinoculation.

Fig. 5. Plum pox virus (PPV) infection of *Arabidopsis* knock-out mutants. **A**, Green fluorescent protein expression foci in leaves of *psag-1.4* and *psak-1* mutants and wild-type (Col-0) *Arabidopsis* inoculated with PPV by agroinfiltration. **B**, Virus accumulation in inoculated leaves, measured by double-antibody sandwich indirect enzyme-linked immunosorbent assay. Each bar represents the mean of five samples assayed in duplicate and each sample consists of four leaves of a single plant. Standard deviation values are shown; dpi = days postinoculation.

system I. In the present study, we have used a similar approach to analyze the role of this gene in PPV infection. Silencing of the *psaK* gene expression enhanced PPV accumulation, suggesting that PSI-K interferes somehow with infection by PPV. In addition to the silencing approach, we analyzed PPV infection in an *Arabidopsis* *psaK* knockout mutant, in which complete depletion of PSI-K is guaranteed (Varotto et al. 2002). Data obtained with the knockout *Arabidopsis* mutant correlated with those obtained in the *N. benthamiana* plants silenced by RNAi, because PPV accumulation was increased markedly when the PSI-K protein was absent, at least in the inoculated leaves. However, systemic infection levels were highly variable in both the wild-type plants and the knockout mutants, preventing us from drawing confident conclusions from the comparative analysis of systemically infected tissues. It is important to remark that differences in infection efficiency could not be detected between wild-type plants and the other mutant analyzed (*psag-1.4*), demonstrating the specificity of the effect observed in the *psak-1* mutant, because both PSI-K and PSI-G proteins are evolutionarily related and share 30% amino acid identity in *Arabidopsis* (Jensen et al. 2002).

Significantly, coexpression of PPV CI drastically reduced the accumulation of PSI-K transiently expressed in *N. benthamiana* leaves (Fig. 6A). This result precluded our original goal of using coimmunoprecipitation or pull-down assays to confirm the in planta interaction between CI and PSI-K proteins. The negative effect of CI expression on the accumulation level of PSI-K was highly specific, strongly suggesting that the two proteins do, indeed, interact in planta. In addition, this result might give us an insight into the possible role of CI-PSI-K interaction in the defense against virus infection. In a first intuitive scenario, CI-PSI-K interaction could interfere with viral infection by inhibiting CI functions. Alternatively, PSI-K could play an antiviral role at a yet unidentified level, and PSI-K degradation, promoted by a CI-PSI-K interaction, would inhibit this antiviral role. In this second scenario, the effect of PSI-K would be exerted predominantly at early infection times, when there are still limiting levels of CI protein. As the amounts of CI increase at later infection times, the effect would tend to disappear, because higher levels of CI would promote a decrease in the available PSI-K protein. This hypothesis correlates well with the results observed in the experiments using RNAi and knockout *psak* mutant plants, in which, at early postinoculation times, PPV accumulation was higher in the absence of PSI-K protein than in control plants, but similar virus titers were observed at later infection times.

Fig. 6. Effect of coexpression of Plum pox virus cylindrical inclusion protein (CI) on accumulation level of transiently expressed PSI-K. **A**, Western blot analysis of extracts (20 μ l) from leaves coinfiltreated with cultures of an *Agrobacterium* sp. transformed with pPSIKcHA, p35S-P1-HC-Pro and pBIN19 empty vector (PSI-K), pNTAPCI (CI+PSI-K), or pNTAPHCPro (HC+PSI-K). Tandem affinity purification (TAP)-tagged CI and HCPro are shown in the top panel and hemagglutinin epitope (HA)-tagged PSI-K is shown in the middle panel. **B**, Western blot analysis of extracts (5 μ l) from leaves coinfiltreated with cultures of an *Agrobacterium* sp. transformed with p35S-green fluorescent protein (GFP), p35S-P1-HC-Pro and pBIN19 empty vector (PSI-K), pNTAPCI (CI+PSI-K), or pNTAPHCPro (HC+PSI-K). TAP-tagged CI and HCPro are shown in the top panel and GFP is shown in the middle panel. Bottom panels of A and B show the equal loading of proteins (Ponceau red stained). Two independent samples (each representing a pool from three independent plants) collected at 6 days after agroinfiltration are shown for each coagroinfiltration mixture.

PSI-K protein is localized in the chloroplast, whereas cylindrical inclusions formed by the CI protein are associated with plasmodesmata at early infection times; later, they accumulate in the cytoplasm (Rodríguez-Cerezo et al. 1997). However, CI-PSI-K interaction still could occur within the replication complexes, because the origin of membrane structures where PPV replication takes place appears to derive from both the endoplasmic reticulum and the chloroplast (Martin et al. 1995). In addition, association of the CP protein from a potyvirus, *Potato virus Y*, with chloroplasts (Naderi and Berger 1997) and the possible interaction of the CP protein from another potyvirus, *Turnip mosaic virus*, with a 37-kDa chloroplastic protein (McClintock et al. 1998), have been described. On the other hand, because CI has been shown to interact with the PSI-K precursor, the possibility exists that PSI-K meets CI before being targeted to the chloroplastic membrane.

PPV infection seemed to negatively affect the accumulation of the PSI-K mRNA in the inoculated leaves. A similar effect has been described previously for other chloroplastic protein mRNAs as a consequence of TMV infection in tobacco *NN* plants (Abbink et al. 2002). In addition, one of these proteins was shown to interact with the helicase domain of the 126K protein of TMV. Moreover, inhibition of photosynthesis has been observed in *Tobamovirus* infections (Rahoutei et al. 2000). All these results support the hypothesis that disturbance of chloroplast protein functions could contribute significantly to induction of defense responses and to the development of disease symptoms.

In summary, the results presented here strongly suggest that chloroplast PSI-K protein interacts with potyviral CI protein, and that PSI-K protein has a negative effect on viral infection. Further experiments are needed to unravel the precise role of PSI-K and to determine whether PSI-K may play its inhibitory role by directly affecting CI functions or through the induction of an unknown defense mechanism, which might be compromised at late infection times when large amounts of CI could target PSI-K for degradation.

MATERIALS AND METHODS

Two-hybrid assays.

The full-length CI sequence was cloned into the bait vectors pLexA and pAS2-1 (Clontech, Palo Alto, CA, U.S.A.) from *Saccharomyces cerevisiae*, as described previously (López et al. 2001). Each vector was cotransformed with a cDNA library from *N. benthamiana* that was kindly provided by L. Jongejan (Leiden University, Leiden, The Netherlands). The cDNA library was constructed in the *EcoRI-XhoI* sites of λ ACTII and contains 1.2×10^6 primary transformants. Automatic subcloning conversion of the cDNA library from lambda to plasmid vectors was done as described previously (Elledge et al. 1991).

Yeast strain L40 was used in the pLex9-CI screening and yeast strain Y190 was used in the pAS-CI screening. Sequential or simultaneous yeast transformations were done using the lithium acetate method (Gietz et al. 1992). Transformants displaying bait to prey interactions were selected on minimal medium lacking histidine, leucine, and tryptophan.

The yeast strain PJ69/4a (James et al. 1996) was used in subsequent two-hybrid assays, and transformants were selected on minimal medium lacking adenine, histidine, leucine, and tryptophan (high stringency), or lacking only the three amino acids (low stringency).

Interaction between murine p53₇₂₋₃₉₀ (pVA3-1) and SV40 large T antigen₈₄₋₇₀₈ (pTD1-1) was used as a positive control in all two-hybrid assays (Clontech).

β -Gal activity was quantified by an in-liquid assay using the luminescent β -gal detection kit II (Clontech). Accumulation

levels of β -gal were estimated on the basis of enzymatic activity of known amounts of purified protein.

Plasmid construction.

Plasmids containing different PPV CI fragments fused to the Gal4 DNA-binding domain have been described previously (López et al. 2001).

The complete nucleotide sequence coding for the TVMV CI protein was amplified by PCR from the TVMV full-length cDNA clone pXBS7 (Domier et al. 1989), using primers 5'-GCGGATCCAGAGTCTAGATACAATAG-3' and 5'-GCGTCGACTCACTGGAATCTTACTGCCTC-3' (restriction sites introduced to facilitate cloning and an artificial termination codon are underlined and in bold, respectively). The PCR product was digested with *Bam*HI and *Sal*II and inserted between the corresponding cloning sites of the pAS2-1 vector. The resulting plasmid was named pAS-CITVMV.

The *N. benthamiana* *psaK* complete cDNA sequence was obtained using the SMART RACE cDNA Amplification Kit (Clontech). The RACE product was cloned into the pGEM-T vector (Promega Corp., Madison, WI, U.S.A.). The *Arabidopsis* PSI-K coding sequence was amplified by reverse-transcriptase PCR from total RNA with primers 5'-GGAATTCGAATGGCTAGC-ACTATGATG-3' and 5'-CCGCTCGAGTCAAATAGCACCAATG-3' (restriction sites introduced to facilitate cloning and an artificial termination codon are underlined and in bold, respectively). The PCR product was digested with *Eco*RI and *Xho*I and inserted between the corresponding cloning sites of the pACT2 vector. The resulting plasmid was named pACT-AtPSIK.

To silence *psaK* gene expression in *N. benthamiana*, an inverted repeat construct was designed. First, the intron I of the ST-LS1 gene of potato (Vancanneyt et al. 1990) was amplified from the intron-containing plasmid pIC-PPV (López-Moya and García 2000) with primers 5'-CACTAGTCCCGGGTAAGTTTC TGCTTCTACC-3' and 5'-GGTACCTGCATATCAACAAATTT TGGTC-3' (restriction sites introduced to facilitate different cloning steps are underlined). The amplified product was digested with *Kpn*I and inserted into the plant expression vector pROK II digested with *Kpn*I and *Sma*I. This vector is based on pBin19 (Frisch et al. 1995) and includes the 35S promoter from *Cauliflower mosaic virus* and the nopaline synthase terminator signal. The resulting plasmid was used to clone a 320-nt fragment of the *psaK* cDNA flanking the intron sequence in both sense and antisense orientation (Fig. 3A). This fragment (nucleotides 160 to 464 of the PSI-K cDNA sequence) was amplified by PCR from the *N. benthamiana* library plasmid pACT-PSIK with primers 5'-CAAGGATCCTTGGGAGCTCGCTG-3' and 5'-GGGTACCAACAGAGATAGATC-3' (restriction sites introduced to facilitate cloning are underlined). The PCR product was digested with *Kpn*I and *Sac*I and inserted between the corresponding cloning sites of the intron-containing pROK II plasmid. The resulting plasmid then was digested with *Spe*I, blunted with Klenow, and digested with *Bam*HI, to allow insertion of the same PCR product digested with *Bam*HI, yielding pdsPSIK.

The GATEWAY technology (Invitrogen, Carlsbad, CA, U.S.A.) was used to construct the tagged PPV CI and *N. benthamiana* PSI-K expression plasmids. The PPV CI coding sequence was amplified by PCR from pIC-PPV using as primers 5'-ggggacaagttgtacaaaaagcaggctTGGAGAGCTTGGACGAT ATAGAAG-3' and 5'-ggggaccactttgtacaagaagctgggtcgATCT GCAGTCATTGATGGTGC-3' (the GATEWAY recombination sites are in lower case). The *N. benthamiana* PSI-K coding sequence was amplified from pACT-PSIK using as primers 5'-ggggacaagttgtacaaaaagcaggctCACATTTCCATTTCTTTGAA ATTTC-3' and 5'-ggggaccactttgtacaagaagctgggtcgAGAGCA CCAATATTCTTGAGGC-3'. The pDONR-207 vector (Invitro-

gen) was used as donor plasmid. Plasmids NTAPi (Rohila et al. 2004) and pGWB14 (Nakagawa T., *unpublished result*) were used as destination vectors to generate plasmids expressing CI labeled with a TAP tag at its N-end and PSI-K labeled at its C-end with three repetitions of the influenza HA, respectively. The BP clonase reactions to introduce the PCR fragments into entry vectors and the LR clonase reactions to transfer the DNA fragments from entry vectors to expression vectors (pNTAPCI and pPSIKcHA) were carried out according to the manufacturer's instructions (Invitrogen). The expression vector Pntaphc-Pro coding for TAP-tagged PPV HCPro was constructed using a similar strategy (A. Valli and J. A. García, *unpublished results*), and p35S-P1-HC-Pro, a plasmid encoding the P1-HCPro silencing suppressor of TEV, has been described previously (Delgadillo et al. 2004). The GFP expression plasmid (35S-GFP) was provided by D. Baulcombe (Sainsbury Laboratory, Norwich, U.K.) (Voinnet et al. 1999).

Plant growth conditions and virus infection.

Plants were grown in a greenhouse maintained at 16 h of light with supplementary illumination at 19 to 22°C.

The recombinant virus PPV-NK-GFP was used in these experiments (Fernández-Fernández et al. 2001). Young *N. benthamiana* plants were inoculated mechanically by rubbing fresh extract of previously infected plants (1 g in 2 ml of 5 mM sodium phosphate buffer, pH 7.4) onto three leaves dusted with Carborundum. *Arabidopsis thaliana* ecotype Columbia (Col-0) and the *psag-1.4* and *psak-1* (Col-0 background) mutants (Varotto et al. 2002) were inoculated by infiltration with *Agrobacterium tumefaciens* C58C1 carrying pBINPPV-NK-GFP, which contains a full-length cDNA copy of the genome of PPV GFP cloned into pBin19 (C. Lucini, J. J. López-Moya, J. M. Alamillo, and J. A. García, *unpublished results*). The agroinfiltration procedure was carried out as described above.

Spread of PPV-GFP was assessed by observation of the GFP fluorescence under a MZ FLIII fluorescence stereomicroscope (Leica, Wetzlar, Germany), using excitation and barrier filters at 480/40 and 510 nm, respectively. PPV accumulation was quantified by double-antibody sandwich indirect ELISA, using the REALISA Kit (C.C. Durviz S. L., Valencia, Spain). Samples were prepared by grinding the infected leaves with mortar and pestle in 5 mM sodium phosphate buffer, pH 7.4 (2 ml/g). A standard curve was obtained with different amounts of purified PPV virions diluted in extract of healthy plants. The enzymatic reaction was developed with the SIGMA FAST p-Nitrophenyl phosphate tablet sets system (Sigma-Aldrich, St. Louis) and the optical density at 405 nm (OD₄₀₅) of the reaction product was measured in a spectrophotometer Multiskan MCC/340 (Titertek, Huntsville, AL, U.S.A.).

Transient silencing assays and plant transformation.

pdsPSIK was used for both transient and constitutive silencing approaches. For transient silencing assays, *N. benthamiana* plants were infiltrated with *A. tumefaciens* C58C1 strain carrying pdsPSIK. Approximately 150 µl of the *Agrobacterium* cultures (OD₆₀₀ = 0.5) induced with acetosyringone were applied with a syringe to the underside of three leaves of young *N. benthamiana* plants (English et al. 1997).

The same *A. tumefaciens* C58CI strain carrying pdsPSIK was used to transform *N. benthamiana* leaf disks as described previously (Horsch et al. 1985). Transgenic plants were regenerated on a selection medium containing kanamycin (100 µg/ml). Rooted plantlets were transferred to soil and maintained in a greenhouse. Seeds from T₀ plants were harvested and sown in a selection medium containing kanamycin to analyze segregation. Seeds from some T₁ plants showing a 3:1 segregation were harvested and germinated in the presence of kana-

mycin to identify homozygous T₁ plants. *PsaK* gene silencing was tested by Northern-blot analysis.

In planta production

of tagged proteins and Western blot assays.

A. tumefaciens C58C1 strains transformed with plasmids expressing TEV P1-HCPro, TAP-tagged PPV CI or HCPro, HA-tagged *N. benthamiana* PSIK, or GFP were handled as described above. Coexpression assays were done by mixing, just before the agroinfiltration, pairs of *Agrobacterium* cultures expressing the appropriate tagged proteins, plus a culture of *Agrobacterium* transformed with the plasmid expressing the untagged silencing suppressor P1-HCPro from TEV (each culture at OD₆₀₀ = 1.5). Infiltrated leaves were harvested at the indicated times, ground to fine powder under liquid nitrogen, and stored at -80°C until use.

Protein extracts were prepared by thawing the powder in extraction buffer (150 mM Tris-HCl pH 7.5, 6 M Urea, 2% sodium dodecyl sulfate [SDS], and 5% 2-mercaptoethanol) (2 ml/mg). Samples were boiled for 10 min and cell debris removed by centrifugation at 18,000 × g at 4°C for 10 min. Supernatants were resolved on SDS polyacrylamide gel electrophoresis (12% acrylamide), electroblotted to nitrocellulose membrane (Amersham, Buckinghamshire, U.K.) and subjected to Western blot analysis using anti-HA (gift of C. Santiago and J. M. Casasnovas, CNB, Spain) or anti-GFP monoclonal antibodies (Roche, Basel, Switzerland) at 1/5,000 dilution. Horseradish peroxidase-conjugated sheep anti-mouse (Amersham) was used as a second antibody and immunostained proteins were visualized by enhanced chemiluminescence detection with ECL (Amersham) according to the manufacturer's instructions. The TAP-tagged CI protein could be detected with any of the used antibodies, because the TAP tag contains a Protein A domain, which is able to nonspecifically bind to immunoglobulins. Red Ponceau staining was used to check equal loading of samples.

Total RNA extraction and Northern blot analysis.

Total RNA was isolated from leaf tissue by the LiCl precipitation method described by Lagrimini and associates (1990). For Northern blot analysis, total RNA (5 µg) was separated on 1.2% agarose gels containing 6% formaldehyde and transferred to a Nylon Zeta-Probe membrane (BioRad, Hercules, CA, U.S.A.). Blots were hybridized with ³²P-labeled DNA probes specific to the PSI-K or GFP coding sequences, synthesized with Rediprime II random prime labeling system (Amersham).

ACKNOWLEDGMENTS

This work was supported by grants BIO2004-02687 from Spanish MEC and BIO4-CT97-2300 and QLG2-CT-2002-01673 from European Union. I. Jiménez and L. López were recipients of FPI fellowships from the Comunidad de Madrid and Spanish MEC, respectively; A. Valli was the recipient of an I3P fellowship from CSIC-Fondo Social Europeo; and J. M. Alamillo held a postdoctoral contract from the Comunidad de Madrid. We thank Elvira Domínguez for technical assistance, L. Jongejan and John Bol for providing the cDNA library from *N. benthamiana*, C. Varotto for providing the *psak-1* and *psag-1.4* mutants, D. Baulcombe for providing the 35S-GFP expression vector, T. Nakagawa for providing pGWB14, M. Fromm for providing NTAPi, and C. Santiago and J. M. Casasnovas for providing the anti-HA monoclonal antibody.

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