Nicotiana benthamiana Kunitz peptidase inhibitor-like protein involved in chloroplast-to-nucleus regulatory pathway in plant-virus interaction

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Plant viruses use a variety of strategies to infect their host. During infection, viruses cause symptoms of varying severity, which are often associated with altered leaf pigmentation due to structural and functional damage to chloroplasts that are affected by viral proteins. Here we demonstrate that Nicotiana benthamiana Kunitz peptidase inhibitor-like protein (KPILP) gene is induced in response to potato virus X (PVX) infection. Using reverse genetic approach, we have demonstrated that KPILP downregulates expression of LHCB1 and LHCB2 genes of antenna light-harvesting complex proteins, HEMA1 gene encoding glutamyl-tRNA reductase, which participates in tetrapyrrole biosynthesis, and RBCS1A gene encoding RuBisCO small subunit isoform involved in the antiviral immune response. Thus, KPILP is a regulator of chloroplast retrograde signaling system during developing PVX infection. Moreover, KPILP was demonstrated to affect carbon partitioning: reduced glucose levels during PVX infection were associated with KPILP upregulation. Another KPILP function is associated with plasmodesmata permeability control. Its ability to stimulate intercellular transport of reporter 2xGFP molecules indicates that KPILP is a positive plasmodesmata regulator. Moreover, natural KPILP glycosylation is indispensable for manifestation of this function. During PVX infection KPILP increased expression leads to the reduction of plasmodesmata callose deposition. These results could indicate that KPILP affects plasmodesmata permeability via callose-dependent mechanism. Thus, virus entering a cell and starting reproduction triggers KPILP expression, which
leads to downregulation of nuclear-encoded chloroplast genes associated with retrograde signaling, reduction in photoassimilates accumulation and increase in intercellular transport, creating favorable conditions for reproduction and spread of viral infection.

KEYWORDS
plant-virus interaction, chloroplast retrograde signaling, carbon partitioning, potato virus X, plasmodesmata, Kunitz peptidase inhibitor-like protein (KPILP), organelles-nucleus-plasmodesmata signaling (ONPS)

Introduction

Plants are constantly exposed to the environmental stress factors and respond to them by modulating the expression of an orchestra of numerous genes, thus adapting to the external stimuli. A coordinated and generalized plant response to adverse factors cannot do without intercellular communication via plasmodesmata (PD), which are unique plant cell structures that cross cell walls and connect the cytoplasm and endoplasmic reticulum (ER) of neighboring cells, forming a symplast (Lucas and Lee, 2004; Burch-Smith et al., 2011b; Burch-Smith and Zambryski, 2012; Patrick et al., 2013; Sager and Lee, 2014; Tilsner et al., 2016; Brunkard and Zambryski, 2017; Faulkner, 2018; Sager and Lee, 2018; Dorokhov et al., 2019; Sun et al., 2019). Intercellular transport is a very important process in plant life. Its regulation is complex and multilevel. Along with PD-localized factors that participate in PD structural and functional changes in response to different stimuli there is more and more evidence that PD are controlled by signals from other organelles, primarily from chloroplasts and mitochondria (Burch-Smith et al., 2011a; Burch-Smith and Zambryski, 2012; Azim and Burch-Smith, 2020; Ganusova et al., 2020). The coordinated functioning of intercellular compartments, accurate and timely transmission of signal molecules, and changes in the pattern of gene expression both at the transcriptional and translational levels are indispensable for the development, growth, and defense reactions of the whole plant.

More than one billion years ago, two independent endosymbiotic events occurred that led to the emergence of a eukaryotic cell with mitochondria and the ability to photosynthesize (Azim and Burch-Smith, 2020; Pfannschmidt et al., 2020). The plastids of the modern green land plants contain about 3000 proteins, more than 95% of which are encoded by a nuclear genome (Brunkard and Burch-Smith, 2018). Chloroplasts are the organelles that convert light energy and produce photoassimilates. But also they house many metabolic processes and play an important role in plant development, growth, and defense responses (Inaba and Ito-Inaba, 2010). Malfunctioning of the photosynthetic apparatus leads to an increase in ROS production and changes in the cell redox status, which in turn effects the pattern of expression of photosynthesis-associated nuclear genes (PhANGs) (Brunkard and Burch-Smith, 2018; Crawford et al., 2018). Many metabolic processes are carried out in plastids and their products determine the physiological status of the cell as they represent the components of the pathways for signal transduction from plastids to the nucleus. This form of signaling is designated as chloroplast retrograde signaling (CRS) (Burch-Smith and Zambryski, 2012). Currently, the CRS signals are classified into the following groups: biogenic signals that are generated by a plastid during its biogenesis and development; operational signals that are produced by a mature chloroplast in response to the environmental changes that affect its metabolism, these signals aimed to lead to stress adaptation; degradation signals are stress-induced signals from the plastids were degraded or destroyed (Crawford et al., 2018; Azim and Burch-Smith, 2020; Pfannschmidt et al., 2020). By modulating the expression of nuclear genes, CRS regulates not only the physiological status of the entire cell, but also affects intercellular communication. The elucidation of the relationship between ISE2, a chloroplast-resident RNA helicase that is involved in chloroplast RNA processing and translation, and PD permeability regulation became a starting point for the research of the chloroplast-PD interplay. Studies of Arabidopsis thaliana ISE2 mutant of midtorpedo stage embryo have revealed the participation of chloroplasts in the regulation of cell-to-cell transport and PD formation (Kobayashi et al., 2007; Burch-Smith et al., 2011a; Bobik et al., 2017). This mutant is characterized by the increased intercellular traffic of 10-kD fluorescent dextran in the midtorpedo stage embryo and contains both simple and branched PD, while wild-type embryos at this stage of development contain only simple PD and limit dextran intercellular distribution (Kim et al., 2002; Kobayashi et al., 2007). Gene expression analysis of A. thaliana ISE2 mutant embryos revealed changes in the expression of nuclear genes encoding plastid proteins participating in tetrapyrrole synthesis, Calvin–Benson cycle and photosynthetic electron transport chain components as well as genes with functions in cell wall
biogenesis and modification (Burch-Smith et al., 2011a). Virus-induced gene silencing (VIGS) of ISE2 in Nicotiana benthamiana leaves led to severe chlorosis, activation of the intercellular transport and secondary PD formation (Burch-Smith and Zambryski, 2010; Burch-Smith et al., 2011a). The interconnection between organelles’ functioning, nuclear gene expression and regulation of PD biogenesis and function underlies the hypothesis of organelle-nucleus-PD signaling (ONPS). It suggests that chloroplasts affecting carbon partitioning (carbon metabolism and translocation of the photoassimilates to the growing point) are the key regulators of all life processes and defense reactions (Burch-Smith et al., 2011a; Azim and Burch-Smith, 2020).

Plant viruses exploit the host plant cell using its resources. Viral infection leads to the depletion of the plant cell, since this process is extremely energy-consuming. Efficient viral propagation and systemic spread requires intracellular reproduction, intercellular trafficking via PD and long-distance transport via vasculature. To succeed, the virus “follows” certain strategies of the host exploitation. Viral infection often leads to the structural modification of cellular components especially membranous compartments. Virus-induced vesicular structures originating from nucleus, ER, peroxisomes, mitochondria or chloroplasts are used for viral replication complexes formation and/or transport (Laliberté and Sanfaçon, 2010). Viral proteins enter these compartments and take control of the organelles’ functioning, modulate the expression of nuclear genes, suppress antiviral immunity to create favorable conditions for reproduction and spread using components and resources of the host cell (Caplan et al., 2008; Zhao et al., 2016; Bhattacharyya and Chakraborty, 2018). Many viruses have chloroplast-targeting proteins encoded in their genome and/or use host cell proteins to reach the chloroplast (Qiao et al., 2009; Li et al., 2016; Bhattacharyya and Chakraborty, 2018; Souza et al., 2019). Exploitation of the cellular membrane compartments is advantageous for viral propagation: chloroplasts isolated by double membrane from the intracellular environment as well as virus-induced membrane structures (virosplams) can serve as a perfect site for formation of viral replication complexes (VRC) protecting viral RNA from the plant cell silencing machinery that functions in the cytoplasm (Tabler and Tsagris, 2004; Laliberté and Sanfaçon, 2010; Bhattacharyya and Chakraborty, 2018). Symptoms of a viral infection are often associated with chlorosis, mottle or mosaic of infected leaves are a consequence of structural and functional changes of chloroplasts caused by viral proteins directly attacking chloroplasts and/or interacting with chloroplast proteins (Reinero and Beachy, 1989; Qiao et al., 2009; Bhat et al., 2013; Li et al., 2016; Budziszewska and Obrepska-Steplowska, 2018). Viral infection leads to suppression of the chloroplast photosynthetic functions and activation of genes related to defense reactions including jasmonate and salycilate pathways (Reinero and Beachy, 1989; Bilgin et al., 2010; Souza et al., 2019).

Plant viruses induce numerous modifications in host gene expression pattern. Despite the accumulated data on plant-virus interactions and transcriptomic changes in response to viral infection, the mechanisms by which viruses affect host gene expression patterns are still not fully understood. Besides genes encoding components associated with defense reactions virus invasion leads to upregulation of the multiple genes the function of which is to be elucidated and the role for the viral propagation is unknown. Among these genes is recently identified Nicotiana benthamiana stress-induced gene encoding Kunitz protease inhibitor-like protein (KPILP) with unknown function (Sheshukova et al., 2017). Its expression is very low in intact leaves and increases in response to tobacco mosaic virus (TMV) infection. KPILP mRNA level correlates with TMV accumulation and chloroplast dysfunction. Moreover, this gene is highly expressed in roots and is activated in leaves in response to prolonged darkness (Sheshukova et al., 2017; Sheshukova et al., 2018). Despite KPILP contains a KPI domain it was shown to have no protease inhibitor activity that could be explained by the lack of amino acid residues that are indispensable for this functional activity (Sheshukova et al., 2017).

Here we study KPILP function and its role in the viral infection. We showed that potato virus X (PVX) systemic infection induces KPILP expression in Nicotiana benthamiana plants up to 9-fold compared to the intact plant. PVX infection activates CRS pathway as was demonstrated by the analysis of photosynthesis-associated nuclear-encoded genes (PhANGs) and CRS marker genes expression that is downregulated. Due to general photosynthesis suppression the level of glucose decreases approximately 4-fold in source and 2-fold in sink leaves compared to the intact plant. To understand KPILP role in PVX-chloroplast interaction we used virus-induced gene silencing (VIGS) approach for KPILP knockdown and demonstrated that KPILP suppression during PVX infection prevents CRS marker genes downregulation and suppression of photosynthetic function. Thereby, photoassimilates level in sink leaves remains the same as in the intact plant. As for source leaves, glucose content is 3-fold higher in leaves with KPILP knockdown compared to PVX-infected leaves with upregulated KPILP. Thus, KPILP participates in CRS and glucose metabolism during PVX infection. Moreover, we have shown that PVX-induced KPILP expression is associated with downregulation of PD callose depositions and increased intercellular transport of macromolecules.

Materials and methods

Plant growth conditions

Wild type Nicotiana benthamiana plants as well as ΔXTFT (Strasser et al., 2008) transgenic N. benthamiana plants were grown in soil in a controlled environment chamber under a 16 h/8 h day/night cycle.
Plasmid constructs

For silencing of endogenous KPILP gene, we used a PVX genome-based expression vector. 183 nt KPILP fragment (from 70 to 253 nt of KPILP coding region) was amplified using F2/R2 pair of primers. PCR product was digested with Nrul-Sall enzymes and inserted in pPVX201 vector (Baulcombe et al., 1995) under control of duplicated CP promoter. To obtain pPVX(frKPILP) vector a fragment from the resulting subclone was transferred into PVX-BIN19 (Komarova et al., 2006) via AvrII/SalI sites.

The set of plasmids encoding KPILP N-mutants was obtained via the following cloning steps. The first step was overlap PCR with two (four in the case of the triple mutant) pairs of primers: for 35S-KPILP(N60A) - F1, R1, F8 and R8; for 35S-KPILP(N86A) - F1, R1, F9 and R9; for 35S-KPILP(N136mut) - F1, R1, F10 and R10. 35S-NbKPILP(ACG) plasmid (Sheshukova et al., 2017) was used as a template. The second step was digesting the obtained PCR product with Acc65I and SalI enzymes, the recognition sites of which were Edenta virus 40 T-antigen NLS was assembled in pUC-based promoter and gene encoding translational fusion between RFP and simian virus 40 T-antigen NLS was assembled in pUC-based promoter and gene encoding translational fusion between RFP and simian virus 40 T-antigen NLS was assembled in pUC-based vector a fragment from the resulting subclone was transferred into PVX-BIN19 (Komarova et al., 2006) via AvrII/SalI sites.

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The plasmid 35S-2xGFP//35S-RFP:NLS was constructed in two steps. First, the expression cassette including the 35S promoter and gene encoding translational fusion between RFP and simian virus 40 T-antigen NLS was assembled in pUC-based plasmid pFF19 (Timmermans et al., 1990). Second, the assembled cassette was transferred to pBin19 containing 35S-2xGFP-cassette (Dorokhov et al., 2012) via NheI and ApaI sites.

The oligonucleotide sequences are listed in Table S1.

Agrobacterium tumefaciens strain GV3101 was transformed with individual binary constructs and grown at 28°C in LB medium supplemented with 50 mg/l rifampicin, 25 mg/l gentamycin and 50 mg/l carbenicillin/kanamycin. Agrobacterium overnight culture was diluted in agrobacterium containing pPVX or pPVX(frKPILP) was infiltrated on to the leaf tissue using a 2-ml syringe, after which the plants were incubated under greenhouse conditions.

PVX inoculation

N. benthamiana plants were inoculated by pPVX or pPVX (frKPILP) via agroinfiltration of the lower leaves and in 10-14 days the systemic PVX infection was detected in the upper leaves.

GFP and RFP imaging

GFP fluorescence was detected using an AxioVert 200M microscope (Carl Zeiss, Germany) equipped with an AxioCam MRc digital camera. The excitation and detection wavelengths for GFP were 487 nm and 525 nm, respectively; the excitation and emission wavelengths for RFP were 561 nm and 625 nm, respectively. The lower epidermal cells from injected leaves were analyzed 20-24 h after infiltration experiments with 2xGFP. Not less than 200 cell clusters per one infiltration area was analyzed and not less than 3 biological repeats per experiment. Three to four experiments were performed.

Callose staining and quantification

To visualize PD-located callose, PVX-infected N. benthamiana leaves with up- or downregulated KPILP, were infiltrated with aniline blue solution (0.1% aniline blue (Sigma Aldrich) in 0.01 M K3PO4 at pH 12). Then the leaves were incubated in the dark at room temperature for 15 minutes before imaging using a Nikon C2 laser scanning confocal microscope. The excitation and emission wavelengths for aniline-blue-stained callose were 403 nm and 447 nm, respectively. Quantification of callose fluorescence was performed as described by Zavaliev and Epel (2015).

Protein extracts preparation

Total soluble protein from PVX-infected leaves was extracted by homogenization of leaf fragment in 1× PBS buffer followed by centrifugation (16,000 × g 10 min) for extract clarification.

The samples from 35S-KPILP-agroinfiltrated leaves were harvested 3 dpi and KPILP-enriched protein extracts for PNGase F treatment were prepared as follows. 0.5 g of leaf material were ground to powder in liquid nitrogen followed by the addition of 3 volumes of extraction buffer (100 mM Tris, pH 8.0, 0.4 M sucrose, 10 mM KCl, 5 mM MgCl2, 10 mM β-mercaptoethanol and 0.1 mM PMSF). The obtained slurry was filtered through a double-layered Miracloth (Millipore/Merck, USA). The material retained on the filter was collected and washed (30-60 min incubation followed by centrifugation at 1000 × g) 5-8 times with the extraction buffer supplemented with 0.1% Triton X-100. When the pellet lost its green color it was washed with the extraction buffer without Triton X-100 and resuspended in one volume of 100 mM sodium-phosphate buffer (pH 7.5).
PNGase F treatment

The KPILP-enriched protein fraction was obtained from the agroinfiltrated *N. benthamiana* plants. PNGase F (New England Biolabs, USA) treatment was performed according to the manufacturer’s protocol under denaturing conditions.

Western blot analysis

Aliquots from protein extracts were analyzed by SDS-polyacrylamide gel electrophoresis and blotted onto polyvinylidene difluoride membranes (GE Healthcare, USA). For KPILP detection, the membranes were probed with polyclonal antibodies against KPILP-6His raised in rabbit (Almabion, Russia). Anti-rabbit antibodies conjugated with horseradish peroxidase (Rockland Immunochemicals, USA) were used as secondary antibodies. The bands were visualized using a chemiluminescence ECL kit (GE Healthcare, USA) and X-ray film.

Measurement of glucose content in plant tissues

Glucose content was assessed as described earlier (Sheshukova et al., 2017). Briefly, dried leaf samples (30 mg) were hydrolyzed with 1 ml of 1M hydrochloric acid (100°C for 2.5 hours). The resulting solution was centrifuged for 10 minutes at 14,000 g. 0.5 ml of the supernatant was diluted with 1.5 ml of water and loaded to a reversed-phase concentrating cartridge (Diasorb C16), the first 1.8 ml was discarded and the next 0.2 ml was collected. Glucosamine solution (1 g/L) was used as an internal standard for each sample. Mixture of equal volume (20 µl) of standard and sample was evaporated on a SpeedVac vacuum centrifugal evaporator with heating. 20 µl 0.5 M solution of PMP (1-phenyl-3-methyl-5-pyrazolone) in methanol and 20 µl of 0.3 M KOH were added to a dried sample and incubated at 70°C for 2 hours. The sample was neutralized by the addition of 20 µl of 0.3 M hydrochloric acid and the excess of the PMP reagent was extracted twice with benzene. The residue was evaporated on a SpeedVac with heating and dissolved in acetonitrile/water (1:9).

The test mixture and analytical samples were analyzed by reversed-phase HPLC in a gradient mode on a Luna C18 (2) 4.6x250 mm (5 µm) column with the mobile phase A – water, B – acetonitrile and D – 100 mM potassium hydrogen phosphate in water (pH 9.12) at a flow rate of 1 ml/min, a temperature of 25°C and with UV detection at 260 nm using a gradient chromatograph Agilent 1100 with PDA detector. Collection and processing of chromatograms was carried out with ChemStation (Agilent) and AutoChrom1200 (ACDlabs) programs.

Quantitative real-time PCR analysis of transcript concentrations

Total RNA was extracted from plant tissues using the TriReagent (MRC, USA) according to the manufacturer’s instructions. The synthesis of first strand cDNA, followed by real-time qPCR was performed as described in (Dorokhov et al., 2012). Briefly, 0.1 mg of random hexamers and 0.1 mg of oligo-dT primer were added to 2 mg of total RNA to obtain cDNA by reverse transcription performed using Superscript IV reverse transcriptase (Invitrogen, Waltham, MA, USA) according to the manufacturer’s protocol. Real-time quantitative PCR was carried out using the iCycler iQ real-time PCR detection system (Bio-Rad, Hercules, CA, USA). Reference genes were detected using the primers to 18s rRNA gene and protein phosphatase 2A gene (PP2A); target genes were detected using sequence-specific primers and Eva Green master mix (Syntol, Russia) according to the manufacturer’s instructions. Primers used for qRT-PCR are listed in the Table S2. Each sample was run in triplicate, and a nontemplate control was added to each run. A minimum of five biological replicates were performed. The qRT-PCR results were evaluated using the Pfaffl algorithm (Pfaffl, 2001).

Statistical analysis

The data was first analyzed for the normality of distribution (Shapiro-Wilk test) and homogeneity of variances (Levene test). The set of values satisfied the Shapiro-Wilk test for the normality of distribution were analyzed by one-way ANOVA. The significance of difference between groups were further assessed using Tukey’s honestly significant difference (HSD) test at p < 0.05 level. For the data that did not satisfy normality of distribution, Kruskal-Wallis non-parametric test was applied. The pairwise comparisons between groups was then performed by *post-hoc* Dunn’s test with Bonferroni correction. In all histograms, y-axis error bars represent the standard error of the mean values.

Results

PVX infection leads to KPILP mRNA accumulation

In intact mature *N. benthamiana* leaves KPILP mRNA accumulation is suppressed due to the expression of a nested alternative open reading frame. But the reproduction of viral vector crTMV-GFP based on the genome of crTMV induces a significant increase in the level of *KPILP* mRNA. Systemic TMV infection of *N. tabacum* was also characterized by intensive
KPILP accumulation, especially in the yellow-green mosaic areas enriched with TMV particles (Sheshukova et al., 2017).

To understand whether KPILP induction is a part of a general antiviral response rather than only a reaction to tobamoviral infection we inoculated N. benthamiana plants with potato virus X (PVX) using a viral vector pPVX [PVX-BIN19 (Komarova et al., 2006)] encoding PVX infectious copy (Figure S1A, left). We observed symptoms of the systemic infection in the upper leaves of the plants 10-14 days after agroinfiltration (Figure S1B, left). We assessed KPILP mRNA content in systemic leaves using quantitative real-time PCR (qRT-PCR) approach and revealed 9-fold increase in KPILP expression level in plants infected with pPVX compared to the intact plants (Figure 1A).

Thus, KPILP mRNA accumulation is elevated in response to PVX infection.

KPILP regulates nuclear encoded chloroplasts genes expression in CRS pathway

Earlier it was demonstrated, that KPILP mRNA level is high in roots and upregulated in leaves after prolonged plant incubation in the dark (Sheshukova et al., 2017). Together with the fact that photosynthesis is suppressed during productive viral infection these allows to suggest that KPILP could be associated with chloroplast functioning or photosynthesis.

To elucidate KPILP role in virus-host interaction we performed KPILP knockdown in N. benthamiana plant using PVX-based vector for virus-induced gene silencing (VIGS) approach (Burton et al., 2000; Shimizu et al., 2009). We constructed a pPVX(frKPILP) viral vector that contained a 183-nt KPILP fragment under control of a duplicated CP subgenomic promoter (Figure S1A, right). N. benthamiana leaves were inoculated by pPVX(frKPILP) via agroinfiltration and in 10-14 days the systemic PVX infection was detected in the upper leaves (Figure S1B, right). The level of viral reproduction in plants with pPVX(frKPILP) or PVX systemic infection was not significantly different as was demonstrated by assessment of PVX genomic RNA levels (Figure S2A) but coat protein accumulation was slightly lower in pPVX(frKPILP) plants (Figure S2B). We analyzed KPILP mRNA accumulation level in upper leaves of intact plants and plants inoculated with pPVX (frKPILP) or pPVX and demonstrated that KPILP was downregulated by 90% in silenced plants compared to PVX-infected plants and decreased to the levels characteristic of the intact plant of the same age (Figure 1A). Thus, we developed a model system that allows to compare effects of KPILP up- and downregulation during PVX infection.

We hypothesize that KPILP is involved in the regulatory pathways for the transmission of signals from chloroplasts to the nucleus. To test this assumption, we assessed the expression of the listed below genes which are responsive to CRS, i.e. the changes of their expression indicate the launch of the chloroplast retrograde signaling. GOLDEN2-LIKE1 (GLK1) encodes transcriptional factor essential for normal chloroplast development (Fitter et al., 2002). GLK1 expression correlates with photosynthesis-associated genes and it is suppressed when functioning of chloroplasts is impaired (Kakizaki et al., 2009). LHCB 1 and 2 encode the antennae proteins of the photosystem-associated light-harvesting complexes (LHC); RBCSIA encodes one of the isoforms of rubisco small subunit that is involved in antiviral plant immune response (Bhat et al., 2013). Changes in LHCB 1, 2 and RBCSIA expression coordinates retrograde signals that define physiological status of chloroplasts (Susek et al., 1993; Burch-Smith et al., 2011a). HEMA1 is another nuclear gene encoding chloroplast protein, viz glutamyl-tRNA reductase that mediates the first step of tetrapyrrole biosynthesis (Schmied et al., 2011).

The qRT-PCR analysis of the corresponding mRNA showed that in pPVX-infected plant where KPILP is activated the expression of all these genes is suppressed compared to the intact plant. While in pPVX(frKPILP)-infected plants with suppressed KPILP, LHCB1 and LHCB2 are slightly downregulated compared to the intact plant (Figure 1B). Nevertheless, LHCB1 and LHCB2 levels are significantly higher in KPILP-silenced leaves compared to PVX-infected. Moreover, in KPILP-silenced plants PVX infection does not lead to RBCSIA and HEMA1 downregulation: their expression remains at levels characteristic of the intact plants.

In addition, we analyzed the expression of XTH5 gene that belongs to XTH gene family encoding xyloglucan endotransglucosylase/hydrolase. XTH5 gene is playing a vital role in response to specific biotic or abiotic stresses and likely participates in xyloglucan remodeling and turnover during primary cell wall formation (Yokoyama and Nishitani, 2001; Ishida and Yokoyama, 2022). XTH5 expression is regulated by ABC1SIC ACID INSENSITIVE4 (ABI4) and ELONGATED HYPOCOTYL5 (HY5) transcriptional factors that are involved in chloroplast-nucleus regulatory hub and plant development (Xu et al., 2016). We have demonstrated that KPILP mRNA level elevated in response to PVX infection correlates with XTH5 expression while in KPILP-silenced plants XTH5 expression is not affected despite they are also infected with PVX (Figure 1B).

To confirm that KPILP affects CRS marker genes expression we agroinfiltrated N. benthamiana leaves with 35S-KPILP to achieve increased levels of KPILP mRNA (Figure S3) and analyzed abovementioned genes expression. The results of qPCR analysis indicate that 35S-directed KPILP overexpression leads to the suppression of LHCB 1, 2, RBCSIA and HEMA1 mRNA accumulation and stimulation of XTH5 expression (Figure 1C). Noteworthy, agroinfiltration with a control “empty” binary vector pCAMbia1300 per se leads to the increase of KPILP mRNA level (Figure S3) inducing some changes in CRS genes expression (Figure 1C). However, most
FIGURE 1

KPI1P is involved in CRS regulatory hub. (A) Relative amount of KPI1P mRNA in leaves with systemic PVX infection (pPVX or pPVX(frKPI1P)) as determined by qPCR. Mean values and standard error are presented. The data was analyzed using ANOVA. Bars without shared letters indicate significant differences according to Tukey’s HSD at p<0.05. (B) Relative amount of CRS marker genes mRNA in response to PVX infection and KPI1P downregulation as determined by qPCR. Mean values and standard errors are presented. The level of mRNA accumulation for each gene in a control plant was taken as 1. The data was analyzed using Kruskal-Wallis test. Bars with different letters indicate significant difference according to post-hoc Dunn’s test at p<0.05 while bars with shared letter are not significantly different. (C) Relative amount of CRS marker genes mRNA in leaves 3 days after agroinfiltration with “empty” pCambia1300 or 35S-KPI1P as determined by qPCR. Mean values and standard errors are presented. The level of mRNA accumulation for each gene in an intact plant was taken as 1. Bars with different letters indicate significant difference at p<0.05 (ANOVA, Tukey’s HSD), while bars with shared letter are not significantly different.
of these fluctuations are not statistically significant comparing to the intact plant while plasmid-directed KPILP overexpression launched more pronounced changes. Therefore, both viral-induced endogenous KPILP and transiently expressed from the plasmid affect the CRS marker genes in a similar way.

Together these results indicate that during PVX infection KPILP participates in operational retrograde signaling, negative regulation of LHCB1 and LHCB2, RBCC1A and HEMA1 genes expression and is associated with increased XTH5 level.

**KPILP is necessary for maturation of the PsbQ subunit of oxygen-evolving complex during PVX infection**

We analyzed total soluble protein of PVX-infected N. benthamiana leaves with upregulated or silenced KPILP. In protein extracts from systemic leaves of pPVX-inoculated plant and leaves from intact plant we revealed a protein band that was absent in the samples from pPVX(frKPILP)-infected plants (Figure 2). Mass-spectroscopy analysis of the tryptic peptides obtained from this band indicated that it corresponded to the chloroplast oxygen-evolving protein 16 kDa subunit (PsbQ) (Uniprot Q5EFR5). PsbQ plays an important role in the luminal oxygen-evolving activity of photosystem II from higher plants and green algae. However, the analysis of PsbQ mRNA level in three studied variants of plants (Figure 2) showed that PsbQ expression is downregulated both in pPVX- and pPVX(frKPILP)-infected plants in the same extent compared to the intact plant. This indicates that the absence of the detectable amounts of PsbQ on the Coomassie-stained gel in pPVX(frKPILP)-infected plants is not solely a result of mRNA level decrease. We suggested that in KPILP-silenced PVX-infected plants PsbQ synthesis, maturation or targeting are impaired and functional PsbQ 16 kDa protein does not reach chloroplasts and/or degraded.

**KPILP regulates photoassimilates accumulation during PVX infection**

Photoassimilates are the product of photosynthesis, and their physiological distribution throughout the whole plant is necessary for proper growth, maturation, and reproduction. Viral infection negatively affects photosynthesis and leads to the exhaustion of the resources. We assessed the glucose level in sink and source leaves of the intact, pPVX- or pPVX(frKPILP)-infected plants. Source leaves are the mature leaves that produce the main volume of photoassimilates serving as a nutrients supply for the whole plant. The fraction of sink tissues included apical parts of the plant with young leaves less than 1 cm in length, flowers and flower buds. In both pPVX- and pPVX(frKPILP)-infected systemic source leaves we observed decrease in glucose level compared to the intact plants: 6-fold and 2-fold, respectively (Figure 3). This indicates the suppression of photosynthesis and carbon metabolism caused by viral infection. However, in sink tissues of the KPILP-silenced plants glucose level was the same as in intact plants and in PVX-infected plants where KPILP is upregulated glucose content decreased 2-fold (Figure 3).

These results allow us to suggest that KPILP participates in carbon metabolism and partitioning during PVX infection and...
Reduced glucose levels are associated with KPILP virus-induced upregulation.

KPILP regulates callose deposition

Biologically, being a virus-induced gene that plays a role in CRS and carbon partitioning, KPILP according to ONPS hypothesis could affect intercellular transport that changes in response to different stresses. One of the PD regulation mechanisms is modulation of callose depositions at PD. Increase in callose deposition induces the reduction of PD aperture and callose degradation results in PD dilation and activation of intercellular transport. Plant viruses could affect PD permeability to mediate cell-to-cell spread of the infection exploiting cellular factors. To assess the effect of KPILP overexpression on callose-mediated PD permeability regulation we analyzed PD-deposited callose in systemic leaves of pPVX- and pPVX(frKPILP)-infected N. benthamiana plants. Callose was stained with aniline blue (Figure S4) (Zavaliev and Epel, 2015) and quantified (Figure 4). The level of callose depositions in pPVX-infected plants with upregulated KPILP was reduced by 13% compared to the intact plants while leaves with KPILP knockdown demonstrated a 13% increase in fluorescence intensity of stained PD callose. Noteworthy, the number of the fluorescent dots and the mean area of them do not differ among analyzed samples indicating that neither plasmodesmata density nor their morphology significantly changed compared to the control plants. These results show that KPILP is associated with decreased callose levels during viral infection.

KPILP stimulates intercellular transport of macromolecules

To evaluate cell-to-cell communication in leaves with elevated KPILP expression, we used a reporter macromolecule containing a fusion of two copies of green fluorescent protein (2×GFP) (54 kDa), as PD from intact mature source leaves are not permeable to proteins larger than 47 kDa (Crawford and Zambryski, 2000). To obtain individual cells or cell clusters expressing 2×GFP, we diluted an agrobacterial suspension to deliver the 35S-based 2×GFP-encoding plasmid to individual cells located distantly from each other. Although the primary transformed cell is clearly distinguishable by the decreasing fluorescence intensity from the cells to which 2xGFP moved (Burch-Smith and Zambryski, 2010), we created a vector, 35S-2xGFP//35S-RFP:NLS, containing two expression cassettes to mark each primary transformed cell nucleus with RFP fused to the nuclear localization signal (NLS) (Figure 5A) and used it for further experiments. Mature N. benthamiana leaves were examined using fluorescent light microscopy 20-24 h after joint agro-transformation with the 35S-2xGFP//35S-RFP:NLS and 35S-KPILP. Counting the number of epidermal cells
surrounding the initial transformed cell that displayed fluorescence provides a quantitative estimation of 2×GFP movement (Burch-Smith and Zambryski, 2010; Dorokhov et al., 2012). When the PD were "closed", 2×GFP was mainly detected in single cells (Figure 5B). While fluorescent signals were distributed in clusters containing 2, 3 or more cells (Figure 5C) when PD were dilated. In these experiments, we used an "empty" binary vector as a control.

When leaves expressing 35S-2xGFP//35S-RFP:NLS and 35S-KPILP were examined, 43% of the signal was distributed in clusters of 2, 3 or more cells, indicating that 2×GFP cell-to-cell movement was enhanced. Specifically, whereas only 5% of the signal was found in clusters of at least 3 cells in the control leaves, this value increased up to 21% in the presence of KPILP (Table 1). Student’s t-tests confirmed the statistical significance of the differences in the cell-to-cell movement of 2×GFP between the control and KPILP-expressing leaves.

**FIGURE 4**

KPILP affects callose deposition around PD. Relative callose levels at PD as estimated by measurement of aniline blue-stained callose fluorescence intensity in *N. benthamiana* plants with PVX systemic infection and KPILP downregulation compared to the intact plants. The level callose in control plants was taken as 1. Mean values in arbitrary units (a.u.) and standard errors are presented. Bars without shared letters indicate significant differences at p<0.05 (ANOVA, Tukey’s HSD).

KPILP N-glycosylation is indispensable for its ability to activate intercellular transport

We previously suggested that KPILP undergoes glycosylation as it contains an N-terminal signal sequence that directs it to the endoplasmic reticulum/Golgi apparatus and putative N-glycosylation sites at Asn-60, Asn-86, and Asn-136 (Figure 6A) according to a prediction by the NetNGlyc service (http://www.cbs.dtu.dk/services/NetNGlyc/) (Sheshukova et al., 2017). Here, to confirm presence of Asn-linked glycans in KPILP, we used peptide-N-glycosidase F (PNGase F) (Plummer et al., 1984), which cleaves a bond between the innermost GlcNAc and asparagine residues in N-linked glycoproteins, except when the core GlcNAc is α1,3-fucosylated (Shen et al., 2017). This cleavage results in an increase of protein electrophoretic mobility. Therefore, to test the sensitivity to PNGase F, we used KPILP-enriched preparations of membrane proteins obtained from wild-type (WT) *N. benthamiana* plants and transgenic ΔXTFT *N. benthamiana* plants with knocked out β-1,2-xylosyltransferase (ΔXT) and α-1,3-fucosyltransferase (AFT) genes. The PNGase-treated samples were analyzed by Western blotting with antibodies against KPILP. KPILP-enriched protein preparations from *N. benthamiana* WT and ΔXTFT plants did not differ in sensitivity to PNGase F (Figure 6B), thus for further PNGase F treatments we used preparations from the WT *N. benthamiana* plants. Western blot demonstrates presence of at least 4 KPILP bands in the gel, which, after treatment with PNGase F, turn into a single major band with lower molecular weight that matches by the mobility to the protein band of the recombinant 6His-KPILP produced in *E. coli* (Figure 6B). This indicates that KPILP is a glycoprotein containing N-linked glycans sensitive to PNGase F.

At the next stage of the study, we created genetic constructs containing KPILP sequence with point mutations that abolished either potential KPILP N-glycosylation site. In the KPILP
(N60A) and KPILP(N86A) mutants, the corresponding asparagine residue was substituted with alanine, while in the KPILP(N136mut) variant, we changed the amino acid context of Asn136 to prevent glycosylation. Moreover, we also created a triple mutant (3Nmut). Then, proteins isolated from N. benthamiana leaves agroinfiltrated with the described plasmids were treated with PNGase F and analyzed by Western blot with antibodies to KPILP (Figure 6C). Deleting the N-glycosylation sites N-60 and N-86 does not completely abolish KPILP glycosylation but changes the electrophoretic profile (Figure 6C). The simultaneous elimination of the three sites, including N-60, N-86 and N-136, also does not completely prevent KPILP glycosylation, which suggests possible glycosylation at other potential sites.

To determine how KPILP glycosylation affects its ability to participate in PD permeability regulation, we compared KPILP mutant variants in a test using 2×GFP reporter molecule. Table 1 shows that introducing a mutation into any of the N-glycosylation site leads to a decrease in GFP-containing multicellular clusters to levels similar to control.

| Variant          | 1 cell  | 2 cells | ≥3 cells |
|------------------|---------|---------|---------|
| control          | 86 ± 5.0 (*) | 9 ± 2.7 (*) | 5 ± 2.5 (*) |
| KPILP            | 58 ± 1.5 | 22 ± 1.0 | 21 ± 1.2 |
| KPILP (N60A)     | 76 ± 2.3 (*) | 17 ± 1.3 (*) | 7 ± 1.6 (*) |
| KPILP (N86A)     | 81 ± 2.9 (*) | 15 ± 1.8 (*) | 4 ± 1.3 (*) |
| KPILP (N136A)    | 80 ± 1.1 (*) | 13 ± 0.8 (*) | 6 ± 0.8 (*) |

*The data shown in % of total number of clusters and represent four independent experiments with no less than five biological repeats and 200 cell clusters per sample analyzed. Means and standard error are indicated. *, p <0.05 (Student’s t-test) for statistical significance of the difference between KPILP and other variants.
We concluded that the preservation of the native KPILP glycosylation profile and its secretion via the ER–Golgi pathway is necessary for its performance of PD gating functions.

Discussion

Viral infection induces multiple changes in host plant cells. Among them are significant modifications of photosynthetic activity and chloroplast functioning (Ganusova et al., 2017; Bhattacharyya and Chakraborty, 2018; Souza et al., 2019; Navarro et al., 2021) that are caused directly by the viral proteins (Reinero and Beachy, 1989; Qiao et al., 2009) or indirectly as a consequence of the re-formatting and functional changes in the cell, i.e. side-effects of viral reproduction (Bhat et al., 2013; Li et al., 2016; Budziszewska and Obrepska-Stepłowska, 2018). Also during viral infection, the intercellular transport of macromolecules is upregulated by the viral and cellular factors.

Here, we studied the role of stress-induced KPILP gene in plant-virus interaction. We designed a model system that allows to monitor the effects of KPILP up- and downregulation on the background of PVX infection: KPILP expression increases in response to viral infection per se and to obtain the decreased KPILP mRNA levels during PVX infection we used VIGS approach exploiting PVX-based vector both to create the conditions of viral infection and to induce KPILP silencing. We have demonstrated that KPILP is involved in CRS regulatory hub during viral infection as it participates in the suppression of nuclear encoded chloroplast genes LHCB1 and LHCB2, RBCS1A and HEMA1. Similar effect on the expression of the studied PhANGs was revealed in N. benthamiana plants with reduced ISE2 level (Ganusova et al., 2020) and in the well characterized Arabidopsis mutant ISE2 embryos that are characterized with impaired photosynthesis and are lethal due to the suppression of genes involved in the LHC formation as well as in chlorophyll synthesis and carbohydrates production (Burch-Smith et al., 2011a). However, in our study the level of ISE2 mRNA remained the same either in pPVX- or pPVX(35S-KPILP)-infected plants compared with intact plants (data not shown). Together these results indicate that KPILP is involved in the suppression of photosynthesis and carbohydrate metabolism during viral infection in an ISE2-independent pathway.

Taking into account that KPILP is known to be upregulated in response to prolonged darkness (Sheshukova et al., 2017; Sheshukova et al., 2018) we could suggest that its activation during viral infection engages the same mechanisms that underlie the darkness-induced activation and both pathways are connected with cellular photosynthetic activity and PhANG expression.

During PVX infection the level of produced and accumulated photoassimilates is significantly decreased. Our
results indicate that KPILP participates in glucose partitioning and carbohydrate metabolism in PVX-infected leaves as in KPILP-silenced plants the level of glucose in sink leaves is 2-fold higher than in sink leaves with upregulated KPILP, moreover, in source leaves of KPILP-silenced plants glucose content is 3-fold higher compared to source leaves with increased KPILP expression. These results allow us to conclude that KPILP negatively affects photosynthates accumulation during viral infection suppressing chloroplast nuclear-encoded genes that determine the physiological status of chloroplasts and triggering CRS. Viral reproduction and activation of antiviral defense, such as viral RNA silencing, is a very energy-consuming process (Nagy and Lin, 2020). Metabolic changes occurring in infected tissues lead to the depletion of photosynthetates necessary for the synthesis of new proteins and energy for biosynthetic processes (Zhao et al., 2016). Chloroplasts are not only the source of photoassimilates but also they generate various forms of ROS, which are strategic activators of defense pathways in cells (Kuzniak and Kopczewski, 2020). During viral infection we observe genetic and metabolic changes in chloroplasts associated with the suppression of carbohydrates synthesis. These effects could be a consequence of the redistribution of energy resources from normal metabolism to the activation of plant defense reactions followed by virus-induced suppression of these defense reactions and utilization of the cellular resources for the viral reproduction. Although PVX genomic RNA levels did not differ between analyzed experimental groups (Figure S2A), PVX CP accumulated less efficiently in plants with KPILP silencing (Figure S2B). However, the question about KPILP negative effect on antiviral defense reactions remains open and requires further study.

In PVX-infected plants with KPILP knockdown we observed a decreased amount of mature PsbQ protein, a component of photosystem II oxygen-evolving complex. We suggested that during PVX infection one or several viral proteins interact with PsbQ precursor exploiting its chloroplast transit peptide to enter chloroplast and photosynthetic apparatus while KPILP is essential for this interaction. A similar interaction with chloroplast proteins has been described for PVX CP that interacts with plastocyanin transit peptide and thus enters the chloroplast (Qiao et al., 2009). It is possible that KPILP downregulation leads to less effective penetration of viral protein(s) into the chloroplast which results to milder suppression of the CRS marker genes and carbohydrate metabolism.

Another KPILP function is associated with PD control. We demonstrated that KPILP stimulates intercellular transport of reporter 2xGFP molecules being a positive PD regulator. Moreover, natural KPILP glycosylation is indispensable for manifestation of this function. And during PVX infection KPILP increased expression leads to the decrease in PD callose deposition. We have demonstrated that increased KPILP expression stimulates intercellular transport and correlates with XTH5 expression. XTHs were shown to be upregulated in ISE2 mutants (Burch-Smith et al., 2011a) or in plants with ISE2 knockdown (Ganusova et al., 2020) both characterized with the increased intercellular traffic. The XTH genes comprise a large group of genes the expression of which is organ-specific and depends on the stage of development (Yokoyama and Nishitani, 2001; Ishida and Yokoyama, 2022). The transglycosylase activity of XTH towards xyloglucans has been long believed to contribute to the loosening of the cell wall during cell growth (Cosgrove, 2016). However, the recent data indicate that XTHs participate in adaptation to abiotic stresses, plant-virus interactions and cell wall structures remodeling (Shimizu et al., 2007; Otulak-Koziel et al., 2018; Hrmova et al., 2022; Ishida and Yokoyama, 2022). Noteworthy, the expression of XTHs genes is regulated by retrograde signals in a light-dependent manner. As was demonstrated for A. thaliana, ABI4 transcription factor promotes XTH5 expression, while HY5 inhibits it (Xu et al., 2016). Both these transcription factors participate in photomorphogenesis. Moreover, ABI4 is a key player in retrograde signaling pathways: it binds the promoter of a retrograde-regulated genes and downregulates PhANG in response to signals from aberrantly functioning plastids (Koussevitzky et al., 2007). HY5 regulates hypocotyl cell elongation and photomorphogenesis (Jing et al., 2013). During seedling de-etiolation ABI4 and HY5 have the antagonistic effects on photomorphogenesis (Xu et al., 2016). As genes for both transcription factors are found in Nicotiana sp., the homologues of ABI4 (Alazem et al., 2014) and HY5 (Oh et al., 2018) could probably function as regulators of KPILP expression in a light-dependent manner and in response to virus. In intact leaves both KPILP and XTH5 expression is low but upregulated during viral infection. Thus, we could speculate that KPILP being a secretory protein affects XTH in the cell wall inducing increase of PD permeability via putative interaction of XTHs with the cell wall components, that are involved in the regulation of callose depositions under stress conditions. This could be either enzymes directly modifying callose, for example, callose synthases or beta-glucanases, or some factors regulation callose depositions indirectly (Zavali et al., 2011; Wu et al., 2018).

Viral infection leads to the suppression of the genes encoding essential components of leaf photosynthetic apparatus. On the other hand, chloroplasts are involved in antiviral immune defense (Nomura et al., 2012; Serrano et al., 2016; Medina-Puche et al., 2020). Obviously, the pattern of gene expression in photosynthetic and non-photosynthetic tissues is fundamentally different. Genes active in roots that are not exposed to light are not active in leaves and photosynthesis-related genes are suppressed there. One of the successful strategies for viral infection could be the strategy of “switching off” photosynthetic genes through the induction of expression of root regulatory genes in order to suppress antiviral immunity triggered by retrograde signals from chloroplasts. In addition to affecting the plant immune system, the virus can also modulate the expression of genes that regulate cell-to-cell and long
distance transport in the same way, thus facilitating viral reproduction and spread of the infection. Based on this hypothesis, our results presented in this article are integrated into a model that describes organelle-nucleus-PD signaling (ONPS) through the activation of KPILP expression under conditions of viral infection. Under normal conditions and in the absence of stressful impact, the expression of KPILP is downregulated, chloroplasts transmit biogenic signals to the nucleus, indicating a normal physiological status, carbon metabolism and PD-transport of sugars and other factors occur normally.

In virus-infected leaves KPILP expression is activated and that launches a regulatory pathway in which the expression of some PhANGs downregulated and the expression of genes participating in PD-regulation is triggered. PhANG suppression leads to the decrease of ROS and hormones production by chloroplasts and weakening of the antiviral defense. PDANG upregulation results in an activation of cell-to-cell and, consequently, long-distance transport, creating favorable conditions for reproduction and spread of viral infection.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Author contributions

NE, ES, TK designed and performed most of the experiments, NE, TK analyzed the data, prepared figures; KK obtained genetic constructs; EA assisted in confocal microscopy imaging; VT quantified glucose content; MS performed mass-spectroscopy and protein analysis. NE and TK developed the general concept, supervised the study and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.1041867/full#supplementary-material

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