Chloroplast Phosphoglycerate Kinase Is Involved in the Targeting of Bamboo mosaic virus to Chloroplasts in Nicotiana benthamiana Plants[OPEN]

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The Bamboo mosaic virus (BaMV) is a positive-sense, single-stranded RNA virus. Previously, we identified that the chloroplast phosphoglycerate kinase (chl-PGK) from Nicotiana benthamiana is one of the viral RNA binding proteins involved in the BaMV infection cycle. Because chl-PGK is transported to the chloroplast, we hypothesized that chl-PGK might be involved in viral RNA localization in the chloroplasts. To test this hypothesis, we constructed two green fluorescent protein (GFP)-fused mislocalized PGK mutants, the transit peptide deletion mutant (NO TRANSIT PEPTIDE [NOTP]-PGK-GFP) and the nucleus location mutant (nuclear location signal [NLS]-PGK-GFP). Using confocal microscopy, we demonstrated that NOTP-PGK-GFP and NLS-PGK-GFP are localized in the cytoplasm and nucleus, respectively, in N. benthamiana plants. When NOTP-PGK-GFP and NLS-PGK-GFP are transiently expressed, we observed a reduction in BaMV coat protein accumulation to 47% and 27% that of the wild-type PGK-GFP, respectively. To localize viral RNA in infected cells, we employed the interaction of NLS-GFP-MS2 (phage MS2 coat protein) with the modified BaMV RNA containing the MS2 coat protein binding sequence. Using confocal microscopy, we observed that BaMV viral RNA localizes to chloroplasts. Furthermore, elongation factor1a fused with the transit peptide derived from chl-PGK or with a Rubisco small subunit can partially restore BaMV accumulation in NbPGK1-knockdown plants by helping BaMV target chloroplasts.

Bamboo mosaic virus (BaMV) is a single-stranded, positive-sense RNA virus. The genomic RNA of BaMV contains five open reading frames (ORFs) and is 6,366 nucleotides in length with a 5′ cap and a 3′ poly(A) tail (Lin et al., 1994; Yang et al., 1997). ORF1 encodes a 155-kD replicase comprised of a capping enzyme domain that exhibits S-adenosylmethionine-dependent guanylyltransferase activity (Li et al., 2001a; Huang et al., 2004), a helicase-like domain with RNA 5′-tri-phosphatase activity (Li et al., 2001b), and an RNA-dependent RNA polymerase domain (Li et al., 1998; Cheng et al., 2001). The three overlapping ORFs (i.e. ORF2, ORF3, and ORF4) are known as the triple gene block. They encode for proteins involved in viral movement (Lin et al., 2004, 2006; Vijaya Palani et al., 2006). ORF5 encodes the viral capsid protein (CP), required for virion assembly and viral movement (Cruz et al., 1998).

The genomes of positive-strand RNA viruses are templates for both translation and replication. Viral replication complexes are likely to be assembled using host factors to synthesize the minus-strand RNA and then the plus-strand progeny RNA. Recent studies have shown that host factors play important roles in assembling the viral RNA replication complex, selecting and recruiting viral replication templates, activating the complex for RNA synthesis, and other steps (Ahluquist et al., 2003; Patarroyo et al., 2012). The translation and the minus-strand RNA synthesis of poliovirus are regulated by host poly(C) and poly(A) binding proteins and viral polymerase precursor 3CD (Waggoner and Sarnow, 1998; Herold and Andino, 2001; Walter et al., 2002). A number of host genes required for Brome mosaic virus replication have been identified systemically by the yeast (Saccharomyces cerevisiae) genetic approach (Ishikawa et al., 1997; Kushner et al., 2003; Mas et al., 2006; Gancarz et al., 2011). The same approach was used to identify the host factors involved in the replication of Tomato bushy stunt virus (TBSV; Panavas et al., 2005; Li et al., 2009b). A heat shock protein90 homolog (Huang et al., 2012) and the Nicotiana benthamiana glutathione transferase U4 (NbGSTU4; Chen et al., 2013), were identified to interact with the 3′ untranslated region (UTR) of BaMV RNA and enhanced the minus-strand RNA synthesis at the early replication step. The Ser/Thr kinase-like protein localized on cell membrane facilitates the BaMV intercellular movement (Cheng et al., 2013).
Previously, we have identified two host proteins (i.e., p51 and p43) interacting specifically with the 3’ UTRs of BaMV by using electrophoretic mobility shift assay (EMSA) and the UV cross-linking competition technique. The results of liquid chromatography-tandem mass spectrometry (LC-MS/MS) and BLAST indicate that the protein sequences of p43 and p51 match the chloroplast phosphoglycerate kinase (chl-PGK) and elongation factor1a (EF1a) of Nicotiana benthamiana, respectively (Lin et al., 2007). Phosphoglycerate kinase is an ATP-generating enzyme that acts in the glycolytic, gluconeogenic, and photosynthetic pathways (Banks et al., 1979; McHarg et al., 1999). chl-PGK is encoded in the nucleus and translated to produce a 50-kD precursor protein and is then processed into mature 43 kD in the chloroplast. In a knockdown experiment through virus-induced gene silencing, the reduction of PGK decreased the accumulation of BaMV coat protein (Lin et al., 2007).

Eukaryotic EF1a has been shown to play a role in binding to the tRNA-like structure and upstream pseudoknot in the 3’ UTR of Tobacco mosaic virus to regulate the gene expression and viral replication (Pathak et al., 2008). EF1a has also been involved in the recruitment of viral RNA and has facilitated the replicase complex assembly of TBSV (Pogany et al., 2008). The 3’ UTR of BaMV cannot only bind its replicase but also the EF1a and has been proposed to regulate viral RNA replication (Lin et al., 2007).

In this study, we transiently expressed two mislocalized PGK mutants to study the possible functions of chl-PGK that is involved in viral RNA replication. In addition, we used confocal microscopy to investigate the localization of BaMV RNA. Finally, we provided evidence that the down-regulation of BaMV accumulation in PGK-knockdown plants can be restored by the expression of the BaMV RNA binding protein EF1a that is fused to a chloroplast transit peptide.

RESULTS

PGK Is Involved in the Early Stage of BaMV Replication

Previously, we found that a PGK of N. benthamiana interacted with the 3’ UTR including poly(A) tail of BaMV RNA. Our results showed that the accumulation of BaMV, but not Cucumber mosaic virus, decreased in PGK-silenced plants (Lin et al., 2007). To further study the function of PGK involved in BaMV replication, protoplasts derived from PGK-knockdown plants were transfected with BaMV RNA and the coat protein accumulation was analyzed. Coat protein accumulation decreased to approximately 31% compared with that of GFP-knockdown protoplasts 24 h post inoculation (hpi; Fig. 1A). The same experiment was performed with Potato virus X (PVX). The coat protein accumulation was not significantly different from that of the GFP-knockdown control at 24 hpi (Fig. 1A). To further confirm that PGK is involved in BaMV accumulation, we performed a time course of BaMV inoculation in luciferase (LUC)- and PGK-knockdown protoplasts. The results indicated that a significant reduction of BaMV accumulation in PGK-knockdown protoplasts could be detected as early as 12 hpi (Fig. 1B). The results suggested that PGK is involved in an early step of BaMV infection.

A Dominant Negative Effect on BaMV Accumulation When the Mislocalized PGK Was Transiently Expressed

Based on the peptide fragments derived from the BaMV viral RNA binding protein identified by LC-MS/MS (Lin et al., 2007), the sequence of amino acids matched to the chl-PGK (accession no. CAA88841.1) rather than to the cytoplasmic PGK (accession no. CAA88840.1; Fig. 2). Although strong identity exists...
between the amino acid sequences of cytosolic and chloroplast PGKs, chl-PGK is more likely to be involved in BaMV RNA interaction. To characterize the possible roles of the chl-PGK involved in the BaMV infection cycle, we cloned the full-length chl-PGK from *N. benthamiana*, which is designated as *NbPGK1*. The sequence alignment showed a 98% identity between *N. benthamiana* and *Nicotiana tabacum* PGKs, and the highest variation occurred in the N-terminal transit peptide. Because chloroplast *NbPGK1* seems to play a pivotal role in the early stages of BaMV infection, we proposed that one possible role of chloroplast *NbPGK1* might be to mediate BaMV viral RNA targeting to chloroplast.

To understand the different subcellular localization effects of *NbPGK1* on BaMV infection, we constructed two chl-PGK mutants. In the first mutant (NO TRANSIT PEPTIDE [NOTP]-PGK), the transit peptide was deleted. In the second mutant (NLS-PGK), a nuclear location signal was fused to a chl-PGK derivative that lacked the transit peptide. The NLS sequence was derived from the gene of the *Simian virus 40* (SV40) large T antigen. These PGKs (i.e. NOTP-PGK and NLS-PGK) were fused with the GFP on the C terminus for transient expression experiments. GFP fluorescence was detected in all transiently expressed leaves. Confocal microscopy of intact, living leaf tissue from these transiently expressed plants revealed that chl-PGK-GFP fluorescence colocalized with chloroplast autofluorescence. Transiently expressed NOTP-PGK-GFP and NLS-PGK-GFP proteins might play a dominant role in blocking the endogenous chl-PGK from assisting in the accumulation of BaMV in plants. The same experiment was performed with PVX. The PVX replicase was previously found to be localized in the endoplasmic reticulum (ER) membrane (Bamunusinghe et al., 2009). As expected, the coat protein accumulation of PVX did not demonstrate a significant difference among those transiently expressed plants (Fig. 3C). According to a previous study, *NbPGK1* interacts with the 3' UTR containing the poly(A) tail of BaMV viral RNA (Lin et al., 2007). We now show that transiently expressed mislocalized *NbPGK1* can lead to a dominant negative effect on the coat protein accumulation of BaMV. Together, these results suggest that BaMV viral RNA is brought to the chloroplast by *NbPGK1* and that this is vital for the accumulation of BaMV at an early stage of infection.

**BaMV RNA Localized to Chloroplast**

If *NbPGK1* plays a role in assisting the BaMV RNA targeting to chloroplast in an early step of infection, we should be able to see the viral RNA localized in the chloroplast. To observe BaMV RNA subcellular localization, we applied a technique using the specific interaction of the GFP-fused phage MS2 (MS2) CP with the target RNA containing the MS2-binding sites (Fouts et al., 1997; Valegård et al., 1997). This technique has been used successfully to study the localizations of
mRNA in yeast and viral RNA in plants (Bertrand et al., 1998; Zhang and Simon, 2003).

Firstly, we inserted eight copies of the MS2 sequence (19 nucleotides per copy) into the BaMV genome between ORF1 and ORF2 (detailed information of the cloning strategy is described in “Materials and Methods”), designated as BaMV/(MS2)₈ (Fig. 4A). Secondly, we created a construct to express a fusion protein NLS-MS2-GFP that carried a nuclear localization signal derived from the large T antigen of SV40 whose expression was transported to the nucleus. NLS-MS2-GFP was transiently expressed in

**Figure 3.** Intracellular localization and the effects of transiently expressed wild-type and mislocalized PGKs. A, Full-length chl-PGK fused with GFP (PGK-GFP) and its derivatives, no transit peptide mutant (NOTP-PGK-GFP), and nucleus localized mutant (NLS-PGK-GFP) were transiently expressed in N. benthamiana leaves indicated. The signal of GFP is shown in green; the autofluorescence of chloroplast is shown in red, and the nucleus stained with DAPI is shown in blue. Bar = 10 μm. B and C, The coat protein accumulation in PGK-GFP, NOTP-PGK-GFP, NLS-PGK-GFP, and GFP transiently expressed N. benthamiana leaves. The plants were agroinfiltrated for 3 d and inoculated with BaMV (B) and PVX virion (C). Two days post inoculation, the relative coat protein accumulation levels were measured on western blots. The numbers shown above each bar are the average of the relative levels of coat protein accumulation with the so derived from at least three independent experiments. Asterisks indicate statistically significant differences compared with the group indicated (**p < 0.001).
N. benthamiana leaves by agroinfiltration. After 4 h of infiltration, we inoculated BaMV- or BaMV/(MS2)$_8$-capped RNA transcript onto the NLS-MS2-GFP expressed leaves. The results indicated that the signal of GFP fluorescence colocalized with that of chloroplast autofluorescence and was restricted on BaMV/(MS2)$_8$-inoculated plants (Fig. 4B). Overall these results suggest that BaMV viral RNA is localized to chloroplast in infected cells, possibly for replication.

The Down-Regulation of BaMV in PGK-Knockdown Plants Can Be Rescued by Expressing BaMV RNA Binding Protein Targeting to Chloroplast

To test if the role of NbPGK1 escorting BaMV targeting to chloroplast can be replaced by other chloroplast targeting proteins, we then constructed and expressed a BaMV RNA binding protein with chloroplast transit peptide. The candidate for BaMV RNA binding protein is EF1a, which was previously shown to bind the 3' UTR of BaMV (Lin et al., 2007). To have EF1a target to the chloroplast, we cloned the full-length EF1a from N. benthamiana and fused it with the transit peptide derived from chl-PGK and a GFP at its C terminus for localization. Because NbEF1a with PGK transit peptide (PGKTP-EF1a-GFP) could not target chloroplast efficiently, we constructed another NbEF1a that was fused with the full length of the small subunit of Rubisco (rbcS-EF1a-GFP). The results indicated that rbcS-EF1a-GFP more efficiently targets to chloroplast compared with PGKTP-EF1a-GFP (Fig. 5A). We then inoculated BaMV on PGK-knockdown plants that transiently expressed EF1a, GFP, PGKTP-EF1a, or rbcS-EF1a. The results showed that the accumulation of BaMV in PGK-knockdown plants was approximately 60% that of the control plants (the LUC-knockdown). The transiently expressed PGKTP-EF1a and rbcS-EF1a could rescue the defective accumulation of BaMV in PGK-knockdown plants up to 76% and 94%, respectively (Fig. 5B). The near-full restoration of virus accumulation (94%) suggests that chl-PGK plays no other role in viral targeting and amplification. This role can be assumed by other protein as long as those proteins can bind BaMV RNA and target to chloroplast.

The Minus-Strand RNA of BaMV Was Detected in Chloroplasts

To test whether BaMV RNA targets to chloroplast for replication, the RNA was extracted from the healthy and inoculated leaf tissues and the isolated chloroplasts. The complementary DNA (cDNA) was synthesized with either oligo(dT) or gene-specific primer. The plus- and minus-strand BaMV were PCR amplified with a specific set of primers after cDNA synthesis. The

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**Figure 4.** The localization of BaMV viral RNA in plant cells. A, The genome of BaMV and the construct of mutant BaMV/(MS2)$_8$ are illustrated. B, N. benthamiana leaves were agroinfiltrated to express the chimera protein containing GFP, MS2 coat protein, and nucleus localization signal (NLS-MS2-GFP) for 4 h and transfected with mock, BaMV, and BaMV/(MS2)$_8$ as indicated. The signal for GFP is shown in green, the autofluorescence of chloroplast is shown in red, and the colocalized signal is shown in yellow. Bar = 20 μm.
virion RNA (50 ng) was used as control to test the specificity of the amplification of minus- and plus-strand RNA. The results indicated that virion RNA can only be detected with the plus-strand-specific reverse transcription (RT; using 39dT primer) but not the minus-strand-specific RT (using BaMV+1 primer). The 18S ribosomal RNA (rRNA) was used as control for cytoplasm-specific RNA (Li et al., 2009a), and the chloroplast PSII core protein C (psbC) gene was used as control for chloroplast-specific gene. The results showed that the minus-strand BaMV RNA was detected in both leaf tissue and isolated chloroplast (Fig. 6). The cytoplasmic control 18S rRNA was only detected in leaf tissue, not in the isolated chloroplast. These results suggest that the detection is specific and minus-strand BaMV RNA is present in the isolated chloroplast. This implied that the BaMV replicative intermediate exists in chloroplasts.

**DISCUSSION**

In general, most of the positive-strand RNA viruses can only encode for a limited number of genes. Therefore, they cannot replicate by themselves; they require assistance from hosts. Host factors support viral infection cycles directly or indirectly (Ahlquist et al., 2003; Nagy and Pogany, 2008). Most of the host factors that have been previously studied are involved in either viral RNA replication or viral movement. In a previous study, we identified host factor chl-PGK, which interacts with BaMV viral RNA (Lin et al., 2007). We now provide evidence that chl-PGK acts as a mediator for the transport of viral RNA to the chloroplast. Knocking down the expression levels of chl-PGK reduces the BaMV coat protein accumulation. The two dominant negative PGK mutants in this study (i.e. NOTP-PGK-GFP and NLS-PGK-GFP) were predicted to redirect the BaMV RNA from the chloroplasts...
to the cytoplasm and nucleus, respectively, and showed reduced accumulation of BaMV CP in plants. These results suggest that chl-PGK is shuttling the BaMV viral RNA to the chloroplast and that this is vital for BaMV replication. In an in situ hybridization with BaMV-specific riboprobe on the infected tissue inspected under the transmission electronic microscope, the BaMV RNA could be detected in chloroplast, mitochondria, and nucleus (Lin et al., 1993). Furthermore, in gold-labeling experiment, chloroplasts were the most labeled organelles within

Table 1. Primers used in this study

<table>
<thead>
<tr>
<th>Name of the Primer</th>
<th>Sequence of the Primer</th>
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<tbody>
<tr>
<td>XbaI/PGK52/F</td>
<td>5'-GCGAGTCTCTAGAATGGCATCAGCTACAGCTTCT3'</td>
</tr>
<tr>
<td>PGK1053/R</td>
<td>5'-GCCTTGGCGCTTCTTAGAG-3'</td>
</tr>
<tr>
<td>PGK514/F</td>
<td>5'-TCCCGAGGCTATCCGAACTGC-3'</td>
</tr>
<tr>
<td>BarnHI/T7-PGK1494/R</td>
<td>5'-GCAATGATGGATCCCTAACCCATCTGTC-3'</td>
</tr>
<tr>
<td>BarnHI/PGK52/F</td>
<td>5'-GCAATGATGGATCCCTAACCCATCTGTC-3'</td>
</tr>
<tr>
<td>BarnHI/NOTP/F</td>
<td>5'-GCAATGATGGATCCCTAACCCATCTGTC-3'</td>
</tr>
<tr>
<td>BarnHI/NLS/F</td>
<td>5'-GCAATGATGGATCCCTAACCCATCTGTC-3'</td>
</tr>
<tr>
<td>KpnI/1494/F</td>
<td>5'-GCAATGATGGATCCCTAACCCATCTGTC-3'</td>
</tr>
<tr>
<td>BamHI/MS2/F</td>
<td>5'-GGCTTCTCTAGAAGACTGAGAT-3'</td>
</tr>
<tr>
<td>BglII/MS2/R</td>
<td>5'-GGCTTCTCTAGAAGACTGAGAT-3'</td>
</tr>
<tr>
<td>XbaI/MSLS/MS2/F</td>
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</tr>
<tr>
<td>KpnI/MS2CP/R</td>
<td>5'-GGCTTCTCTAGAAGACTGAGAT-3'</td>
</tr>
<tr>
<td>XbaI/Ef1/2/F</td>
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</tr>
<tr>
<td>XbaI/EF1a/F</td>
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</tr>
<tr>
<td>EF1a-T7-XhoI/R</td>
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<td>EF1a-T7/XhoI/R</td>
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</tr>
<tr>
<td>NdeI/Ef1a/F</td>
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</tr>
<tr>
<td>18SrRNA(+)</td>
<td>5'-GGCTTCTCTAGAAGACTGAGAT-3'</td>
</tr>
<tr>
<td>18SrRNA(–)</td>
<td>5'-GGCTTCTCTAGAAGACTGAGAT-3'</td>
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Figure 6. The minus strand of BaMV RNA was detected in the chloroplasts isolated from the inoculated N. benthamiana. The N. benthamiana plants were inoculated with 3 µg BaMV virion (I) or H2O (H) as indicated on the top of each lane. The total RNAs were extracted from leaf tissue or isolated chloroplast. The cDNAs were synthesized from these RNAs with specific sets of primers for reverse transcription-PCR. The BaMV minus- and plus-strand RNAs were indicated as BaMV(–) and BaMV(+), respectively. The psbC gene is a chloroplast-encoded gene and used as the chloroplast-specific marker. 18S rRNA was used as a cytoplasmic-specific marker. I indicates RNA was processed as above but the reverse transcription step was omitted as a negative control. vBaMV(+) and vBaMV(–) indicate the signal of viral RNA from the viral particles.
Phosphoglycerate kinase is targeting viral RNA to chloroplasts

BaMV-infected cells. The gold labeling was observed throughout the chloroplast (Lin et al., 1993). Similarly, in this study, the GFP labeling in the MS2 experiment was uniform in the chloroplast (Fig. 4). These results suggest that the virus does not simply associate with the chloroplast membrane but that it enters the chloroplast stroma.

Once positive-strand RNA viruses infect a host, they usually target to a specific organelle for replication (Ahliquist et al., 2003; Laliberté and Sanfaçon, 2010). Tobacco etch virus (Schaad et al., 1997), Couvea mosaic virus (Carette et al., 2000), Tomato ringspot virus (Han and Sanfaçon, 2003), PVX (Bamunusinghe et al., 2009), and Tobacco mosaic virus (Kawakami et al., 2004; Nishikiori et al., 2006) target the ER membrane; TBSV (McCartney et al., 2005) and Melon necrotic spot Carmen virus (Mochizuki et al., 2009) target the peroxisome and mitochondria, respectively; and Turnip yellow mosaic virus (Prod’homme et al., 2003) and Turnip mosaic virus (Wei et al., 2010) associate with the chloroplast membrane. These findings suggest that members from different viral families might be associated with the same organelle, but that members of the same family do not necessarily target the same organelle or organellar membrane (Laliberté and Sanfaçon, 2010). Therefore, the fact that BaMV targets chloroplast instead of the ER, where PVX, another member of the Potexivirinae genus, replicates, is perhaps unsurprising (Bamunusinghe et al., 2009). Although viral RNA is commonly associated with specific organellar membranes, the mechanism by which it targets specific organelle requires further study.

By using the well-studied binding mechanism of the MS2 bacteriophage protein coat and the MS2 RNA hairpin structure (Bertrand et al., 1998; Zhang and Simon, 2003), we are able to localize the BaMV genomic RNA in chloroplasts of live N. benthamiana cells (Fig. 4). In a complementation study, by supplying the chimera BaMV binding protein (rbcS-EF1a-GFP) in the PGK-knockdown plants, the accumulation of BaMV was restored (Fig. 5). Furthermore, the minus-strand BaMV RNA representing the replicative intermediate was detected in the chloroplast (Fig. 6). These results suggest that BaMV RNA is hitchhiking by binding to chl-PGK and targeting to the chloroplast for replication. To our knowledge, the interaction between a host protein and viral RNA represents a novel strategy for a plant virus to target to its replication site. Other host proteins have been implicated in targeting viral replication proteins rather than viral RNA to specific organelles. For example, Tobamovirus Multiplication Protein1 interacts with the helicase domain of the tobamovirus replication protein and anchors it to ER membranes (Yamanaka et al., 2000). Peroxinsomal Receptor Protein19p (Pex19p) interacts with the tombusvirus p33 protein and targets it to the peroxisomes (Pathak et al., 2008).

In this study, we discovered a novel strategy used by viral plant RNA to target chloroplasts by interacting with nuclear-encoded chloroplast proteins. Through this interaction, viral RNA could take the advantage of the transit peptide-containing protein to move from the cytoplasm to the chloroplast.

**MATERIALS AND METHODS**

**PGK-Knockdown Protoplast Isolation and Viral RNA Transfection**

*Nicotiana benthamiana* plants were grown in pots at a 28°C growth room under 16 h/8 h light/dark cycle. *Agrobacterium tumefaciens* strains were cultured at optical density at 600 nm of 1, containing pTRV1 or pTRV2 (Ono et al., 2006) and plasmids (pTRV2.GFP, pTRV2.PDS, and pTRV2.PGK-3; Lin et al., 2007) were mixed in 1:1 ratio and infiltrated with a syringe onto three leaves of a plant. Ten days after infiltration, the *phytoene desaturase* (PDS)-knockdown plants were shown a photobleaching phenotype indicating an occurrence of gene silence.

Protoplasts were isolated from PGK- and GFP-knockdown *N. benthamiana* plants. In brief, about 2 g of *N. benthamiana* leaves were digested with 12.5 mL enzyme mixture containing pectinase and cellulase and incubated at 25°C overnight. The cells were collected from the interface of 0.55 m mannitol-MES and 0.53 m Suc solution (Tsai et al., 1999). About 3×10⁶ cells were transfected with 1.5 μg BaMV or PVX viral RNA in 40% (w/v) polyethylene glycol solution. Finally, the protoplasts were resuspended in culture medium (1 μM CaCl₂, 1 μM KI, 10 mM MgSO₄, 0.2 mM KPO₄, 10 mM KNO₃, 10 mM CaCl₂, 0.03% cephaloridine, and 0.001% gentamycin in 0.55 m mannitol-MES) and incubated at 25°C under constant light (Tsai et al., 1999).

**Constructs**

The full-length cDNA of *N. benthamiana* chl-PGK was done by two-step cloning. Firstly, the 5’ or 3’ fragment of PGK was amplified from *N. benthamiana* cDNA using the primers according to the sequences derived from *N. tabacum* PGK full-length cDNA sequence (accession no. Z48977). The primers (Table I) for the 5’ fragment of PGK were XbaI/PCK52/F and PGK5103/R, and the primers for the 3’ fragment of PGK were PGK5314/F and BamHI/T7/PK1494/R. The T7 tag was introduced during PCR and cloned into pcGEM-T Easy vector (Promega). The sequence of the 5’ and 3’ fragments of PGK were confirmed by sequencing and compared with *N. tabacum* PGK cDNA full-length sequence. Secondly, the 5’ and the 3’ fragments were digested by XbaI/Xhol and Xhol/BamHI, respectively. The two fragments were then ligated together and cloned into pUC18 vector.

To create transit peptide deletion PGK mutant (NOTP-PGK) and NLS (from SV40 large T antigen)-fused PGK without transit peptide (NLS-PGK), PCR/pUC18/PK1 plasmid was used as a template for PCR. The primers (Table I) used for constructing mutants were BamHI/PK52/F and KpnI/1494/R for with PGK, BamHI/PGK52/R for NOTP-PGK, BamHI/NLS/F and KpnI/1494/R for NLS-PGK. These amplified fragments were cloned into pGEM-T Easy vector and subcloned into pBf-mGFP vector (kindly provided by Chang-Hsien Yang [National Chung Hsing University]), which was derived from the original vector pBf355-mGFP4 (Haseloff et al., 1997) with BamHI and KpnI sites. GFP protein was then fused to the C terminus of PGK constructs. These constructs were designated as pBf-PGK-GFP, pBf-NOTP-PGK-GFP, and pBf-NLS-PGK-GFP.

The EF1a gene of *N. benthamiana* with 3’-end T7 tag was amplified with primers Xhol/EF1a/F and EF1a-T7/Xhol/R (Table I) and cloned into pBf-mGFP vector by restriction enzyme sites Xhol and Xhol to generate pBf-EF1a-T7. The cDNA of PGK transient peptide was amplified by primers BamHI/PGK52/F and PGKTP/Ndle/R, and the ORF of EF1a with Ndle site was amplified by primers Ndle/EF1a/F and EF1a-T7/Xhol/R. The two DNA fragments were digested with BamHI/Ndle and Ndle/Xhol, respectively, and cloned into pBf-mGFP vector BamHI and Xhol to generate pBf-PGKTP-EF1a-T7. The full-length cDNA of rbcS was amplified with primers Xhol/rbcS/F and rbcS/Ndle/R and then, in combination with the full-length EF1a amplified previously, cloned into pBf-mGFP to generate pBf-rbcS-EF1a-T7. For subcellular localization, the ORF of EF1a was amplified by primers Xhol/EF1a/F and Xhol/1494/R and cloned into pBf-mGFP vector by restriction enzyme sites Xhol and SalI to generate pBf-EF1a-T7. The pBf-PGKTP-EF1a-GFP and pBf-rbcS-EF1a-GFP were created by replacing EF1a-T7 of pBf-PGKTP-EF1a-T7 and pBf-rbcS-EF1a-T7 to EF1a-GFP, respectively.
Transient Expression for Localization of Mutant PGK and for Virus Challenge

The preparation of *A. tumefaciens* containing pBIN-PGK- GFP, pBIN-NOTTP-PGK- GFP, pBIN-NLS-PGK-GFP, pBIN-EF1a-GFP, pBIN- PGKTP-EF1a-GFP, and pBIN-rbcS-EF1a-GFP was described previously (Chiu et al., 2010). Two days after agroinfiltration, the tissues containing transiently expressed proteins were stained with DAPI (Invitrogen) for 15 min. Live cell imaging is performed on an inverted fluorescence confocal microscope (FV1000, Olympus) using a 60× oil objective lens. The 405-nm laser was used to image DAPI, a 488-nm laser was used for GFP imaging, and a 633-nm laser was used to image chloroplast autofluorescence. Images were analyzed by Olympus FV10-ASW 1.3 viewer software and processed using Adobe Photoshop CS.

For the following virus-challenging experiment, the *A. tumefaciens* C58Cs1 strain containing pBIN-PGK-GFP, pBIN-NOTTP-PGK-GFP, pBIN-NLS-PGK-GFP, pBIN-EF1a-GFP, pBIN-PGKTP-EF1a-GFP, pBIN-rbcS-EF1a-GFP, and pBIN-mGFP were each mixed with pBIN61-HePro in 1:1 volume ratio and infiltrated into *N. benthamiana* leaves. Three days later, leaves were inoculated with 1 μg BaMV or PVX virion. Total proteins were extracted for western-blotting analysis at 2 d post inoculation.

Protein Detection

Total protein from protoplasts or leaves were extracted with 1× Laemmli buffer (2.5 mM Tris-HCl, pH 8.3; 50 mM Gly; 0.1% SDS), separated by 12% SDS-PAGE, transferred onto a nitrocellulose membrane, and blocked with 5% nonfat dry milk in Tris-buffered saline. The membranes were probed with antibodies against HA (Roche), GST (Novagen), or PGK (Promega), and then incubated with HRP-conjugated antimouse antibody (Roche). Bands were visualized by ECL (GE Healthcare).

Chloroplast Isolation and Minus-Strand BaMV RNA Detection

The plasts isolation was modified from the described protocol (Cowan et al., 2012). Briefly, approximately 1 g of leaf tissue, harvested from BaMV- or mock-inoculated 3-week-old *N. benthamiana* plants at 3 days post inoculation was ground with 5 mL grinding buffer (0.35 M sorbitol, 2 mM EDTA, 0.5 mM MgCl\(_2\), 50 mM HEPEs-KOH, pH 7.5, 1 mM dithiothreitol, and 0.1% bovine serum albumin) and filtered through Miracloth. The filtrate was centrifuged at 1,000 × g for 5 min, and the pellet was suspended in 1 mL suspension buffer (0.35 M sorbitol, 10 mM K\(_2\)HPO\(_4\), 0.5 mM MgCl\(_2\), 35 mM HEPEs-KOH, pH 8.3, and 1 mM dithiothreitol). The suspended preparation was layered on the Percoll step gradient comprising 40% and 80% Percoll (2 mL each) with the suspension buffer and centrifuged at 13,000 × g for 7 min. The intact plastids were isolated from the interface layer. The isolated plastids were washed three times with 5 mL suspension buffer and centrifuged at 1,000 × g for 5 min each time. The plastids were suspended in 0.4 mL 1 mM mGlu buffer containing 40 units of micrococcal nuclease and 5 mM Ca\((\text{CH}_3\text{COO})_2\), and incubated at 30°C for 30 min. Finally, 80 μL EGTA was added to terminate the micrococcal nuclease activity.

The total RNA was extracted from leaves and isolated chloroplasts with TRIzol Reagent (Gibco) and the cDNA was synthesized with ImProm-II Reverse Transcription System (Promega). The plus-strand RNA of BaMV was detected by reverse transcription with 39T primer and amplification with the primer pair BaMV520f and BaMV1024R. The minus-strand RNA of BaMV was detected by reverse transcription with BaMV+1 primer and PCR amplification with the same primer pair (BaMV520f and BaMV1024R) as those used in detection of plus-strand RNA. psbC gene, as the chloroplast-specific gene, was detected by reverse transcription with psbC/RT primer and PCR amplification with primer pair psbC/F and psbC/R. 18S rRNA was used as a cytoplasmic control and was analyzed by reverse transcription with 18S/RT primer and amplification with 18Sf/RT primer and 18Sb/RT primer. Virion RNA (50 ng) extracted from BaMV viral particle was reverse transcribed with 39Tf or BaMV+1 for detecting the plus- or minus-strand RNA as the positive and negative controls, respectively. The PCR was performed at 95°C for 5 min, followed by 95°C for 30 s, 53°C for 30 s, 72°C for 30 s for 15 cycles, and 72°C for 5 min. The detection of 18S rRNA was at the same condition but only for 10 cycles.

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