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The Identification of *cis*- and *trans*-Acting Elements in the Infection Cycle of *Bamboo mosaic virus*

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ABSTRACT

Bamboo mosaic virus (BaMV) is a single-stranded positive-sense RNA virus with flexuous rod morphology which belongs to the potexvirus group. To study the infection cycle of BaMV at the molecular level, a full-length infectious cDNA clone was constructed for *in vivo* protoplast and plant transfection assays. For *in vitro* transcription assays, RdRp replicase complex extracted from infected plants was used. From the results derived from these *in vivo* and *in vitro* analyses, we identified that a cloverleaf-like structure in the 3′ UTR of the BaMV genome is involved not only in viral RNA accumulation in cells but also in viral systemic movement. A major stem-loop contains two *cis*-acting elements, one is a potexviral conserved hexamer motif which is important for the accumulation of viral products in protoplasts and the other is the polyadenylation motif which is involved in minus-strand RNA synthesis and in regulating the length of the poly(A) tail. To identify the *trans*-acting factors required for efficient accumulation of viral products in plants two techniques were employed. The first one is conventional chromatography to isolate the proteins that bind to the RNA genome. The second is cDNA-amplified fragment length polymorphism (cDNA-AFLP) to identify the host gene expression profiles that show up- or down-regulated patterns corresponding to BaMV infection. We found that chloroplast phosphoglycerate kinase (PGK), a gluconeogenic enzyme binds to the poly(A) of BaMV RNA and using virus induced gene silencing, a powerful tool for functional analysis, we found that chloroplast PGK is required for efficient accumulation of BaMV in plants. Using the combination of cDNA-AFLP and VIGS, we will identify novel genes that regulate the accumulation of viral products in plants.

Keywords: host factors, viral RNA replication, cDNA-AFLP, protein-RNA interaction

CONTENTS

INTRODUCTION

Most cultivated species of bamboo with rhizomes of the pachymorph type in Taiwan are susceptible to bamboo mosaic; especially in green bamboo (*Bambusa oldhamii* Munro) and Ma Chu (*Dendrocalamus latiflorus* Munro). Symptoms include mosaic on the leaves (**Fig. 1**) and brown internal streaking of shoots and young culms (Lin *et al*. 1979). The causal agent is *Bamboo mosaic virus* (BaMV) which was first reported in Brazil (Lin *et al*. 1977). Besides bamboos, BaMV can also be mechanically inoculated onto *Chenopodium quinoa*, *C. amaranticolor*, *Gomphrena globosa*, *Nicotiana benthamiana*, *N. plumbaginifolia*, barley (*Hordeum vulgare* L. cv. 'Larker') and rice (*Oryza sativa*). BaMV has a single-stranded positive-sense RNA genome and belongs to the genus *Potexvirus* of the family *Flexiviridae*. It causes a serious disease with mosaic symptoms (**Fig. 1**) on bamboos in Taiwan. The genome comprising 6,366 nt [excluding the 3′ poly(A) tail] (Lin *et al*. 1994) has 5 open reading frames (ORFs) with a 5′ cap and a 3′

Fig. 1 The mosaic symptoms on bamboo leaves infected with *Bamboo mosaic virus*.

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Invited Mini-Review

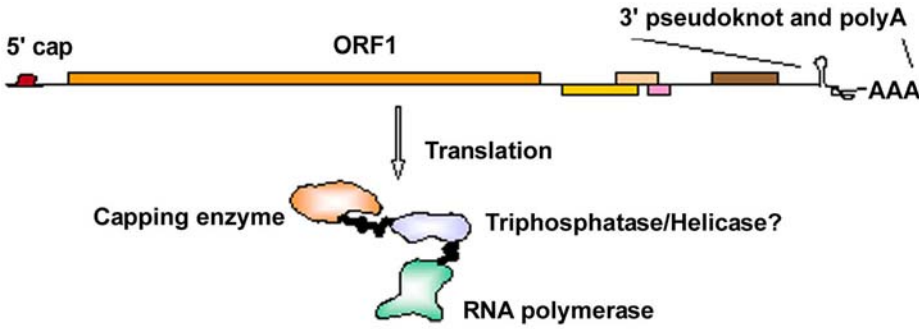

Fig. 2 Illustration of the genome organization of BaMV and constituents of ORF1 product. (Adapted from Tsai *et al*. 2006).

poly(A) tail (**Fig. 2**). The 155-kDa polypeptide of ORF1 has been reported to comprise 3 domains: a capping enzyme domain (Hodgman 1988; Huang *et al*. 2004; Huang *et al*. 2005; Li *et al*. 2001), a helicase-like domain with a RNA 5′ triphosphotase activity (Hodgman 1988; Gorbalenya and Koonin 1989; Li *et al*. 2001), and an RNA-dependent RNA polymerase (RdRp) domain (Li *et al*. 1998). The ORFs 2-4, which are called triple gene block and encode 28, 13, and 6 kDa polypeptides (TGBp1, TGBp2, and TGBp3), respectively, are required for virus movement. The last ORF encodes a 25 kDa capsid protein. Two major subgenomic RNAs of 2.0 and 1.0 kb are 3′ co-termini and are required for the expression of the movement protein (Lin *et al*. 2004) and coat protein (Lin *et al*. 1994), respectively.

At least 2 major steps are involved in the replication of positive-sense RNA viruses: the minus-strand and plus-strand RNA syntheses. The key enzyme catalyzing these reactions is the viral-encoded RdRp (Buck 1996), which may associate with host factor(s) to form a replicase complex (Lai 1998). An *in vitro* transcription system using the purified RdRp complex is a valuable strategy in identifying the host factor(s) in the complex as well as the *cis*-elements of the RNA templates. *In vitro* copying the exogenous templates with a purified, solubilized RdRp complex has been demonstrated in *Brome mosaic virus* (Quadt and Jaspars 1990), *Cucumber mosaic virus* (Hayes and Buck 1990), *Turnip crinkle virus* (Song and Simon 1994), *Tobacco mosaic virus* (Osman and Buck 1996), *Turnip yellow mosaic virus* (Deiman *et al*. 1997; Singh and Dreher 1997) and BaMV (Cheng *et al*. 2001; Lin *et al*. 2005b).

Constructing a full-length infectious cDNA clone or a replicon is another way to understand the functions of the products encoded by each ORF (Beck *et al*. 1991; Chapman *et al*. 1992; Guilford *et al*. 1991), the *cis*-elements required for viral replication (Weiland and Dreher 1989; Tsai and Dreher 1992; White *et al*. 1992), and the relationships between the viral RNAs and host symptom development (Chapman *et al*. 1992; Kavanagh *et al*. 1992).

THE *CIS*-ACTING ELEMENTS IN BaMV INFECTION CYCLE

The *cis*-acting elements reside in the 3′ UTR of BaMV RNA

The *cis*-acting elements for minus-strand RNA synthesis of positive-sense RNA viruses located at the 3′ untranslated region (UTR) have been identified *in vitro* in several plant RNA viruses: the stem-loops of *Alfalfa mosaic virus* (Houser-Scott *et al*. 1997; Houser-Scott *et al*. 1994; Reusken and Bol 1996) and TCV (Song and Simon 1995), and the tRNA-like structures of BMV, TMV, and TYMV (Kao and Sun 1996; Osman and Buck 1996; Deiman *et al*. 1997, 1998; Singh and Dreher 1997). The primary sequence and the secondary structure localized at the 3′ UTR in picornaviral RNAs are presumably recognized by the replicase complex containing viral and host proteins that initiate the minus-strand RNA synthesis (Pilipenko *et al*. 1992; Cui *et al*. 1993; Cui and Porter 1995; Todd *et al*. 1995). Mutations introduced to abolish the pseudoknot structure of the entero-virus RNA are non-viable; however, viability is restored by the compensatory mutations (Mirmomeni *et al*. 1997).

The tertiary structure of the 3′ UTR of BaMV RNA comprising 3 consecutive stem-loops form a cloverleaf-like structure (ABC region), a major stem-loop containing a bulge and an internal loop (D region), and a pseudoknot containing part of the poly(A) tail (E region; **Fig. 3**). The conserved sequences among all members of the potexviruses, the hexamer (ACXUAA) and the putative polyadenylation (AAUAAA) motifs, are located at the apical and internal loops, respectively, in the D region (**Fig. 3**). Results derived from *in vivo* protoplast transfection showed that the hexamer motif is important for the accumulation of viral products in BaMV (Cheng and Tsai 1999) as well as in C*lover yellow mosaic virus* (White *et al*. 1992). Further studies of this motif indicated that the very 5′ adenylate residue of this motif is purine specific, and the following cytidylate is restricted to pyrimidine. The nucleotides at positions 4-6 of this motif, UAA, are unalterable. Substitution at the third position has less effect on viral accumulation in protoplasts (Chiu *et al*. 2002).

A mutation in polyadenylation signal, AAUAAA, of *White clover mosaic virus* or a deletion containing this motif in the 3′ UTR of BaMV RNA reduced the infectivity dramatically (Guilford *et al*. 1991; Kao and Sun 1996). Fifteen single point substitutions introduced in this motif of BaMV RNA revealed that the third nucleotide (the only uridylate residue) is less important in viral RNA accumulation in protoplasts and plants. The second and the last adenylate residues are involved in minus-strand RNA accumulation. However, the two adenylates at the fourth and the fifth positions in this motif are not only involved in minus-strand RNA synthesis but also in polyadenylation efficiency, the length of the poly(A) tail being reduced from 150 to less than 50 adenylates, on average, when mutations are introduced at these 2 positions (Chen *et al*. 2005).

Maintaining the integrity of the structures in D stem-loop and pseudoknot is essential for efficient viral accumulation in protoplasts (Tsai *et al*. 1999). Mutations that disrupt the stem formation in these structures resulted in inefficient accumulation of viral products. However, when compensatory mutations were introduced to reform the stems the accumulation viral products is restored. Full-length transcripts with fewer than 10 adenylates at the very 3′-end fail to accumulate viral products in protoplasts, however, transcripts with 15 or 22 adenylates can reach to 26% or similar to that of the wild type, respectively (Tsai *et al*. 1999). These results suggested that maintaining the pseudoknot structure is important for viral RNA replication. The initiation site of minus-strand RNA synthesis is not fixed at one position but, rather, resides opposite one of the 15 adenylates of the poly(A) tail, with the highest frequency of initiation being from adenylates 7 to 10 counted from the 5′ most adenylates of the poly(A) tail of the 3′ UTR of BaMV genomic RNA (Cheng *et al*. 2002).

The cloverleaf-like ABC region in the 3′ UTR is involved in not only the accumulation of viral products in protoplasts and plants but also viral long-distance movement (Chen *et al*. 2003). The accumulation of viral products of mutants BaMV40A/ΔB and -/ΔC were about 30% that of

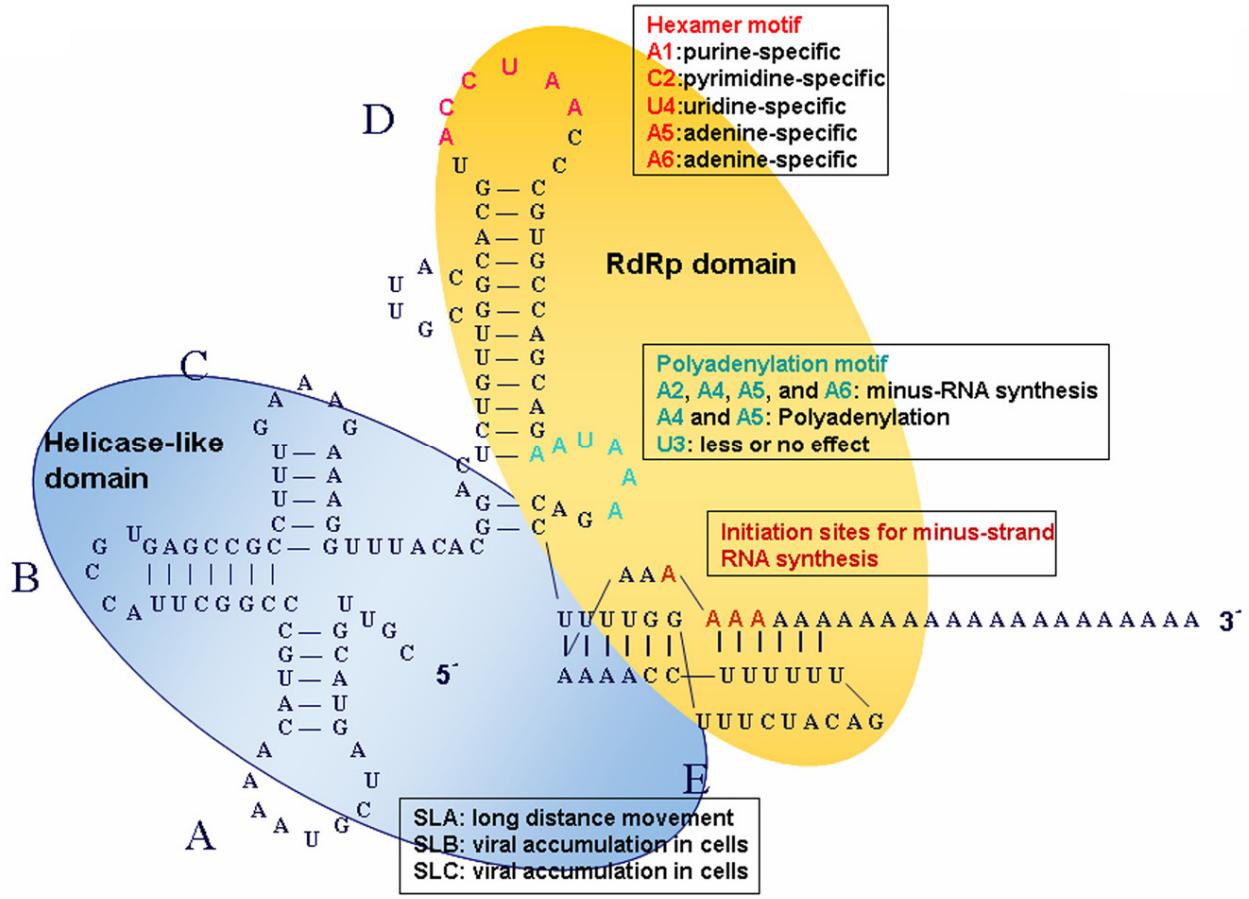

Fig. 3 Illustration of the interactions of the BaMV 3′ UTR and two of the three domains of its ORF1 (adapted from Tsai *et al.* 2006). The RdRp domain shown in yellow and the helicase-like domain shown in blue with oval shape are indicated. The functions of the motifs and *cis*-acting elements in the 3′ UTR of BaMV RNA are indicated in boxes. SL stands for stem-loop.

the wild type. However, the level of accumulation of viral products of BaMV/ΔA was similar to that of the wild type in protoplasts and inoculated leaves. Interestingly, the accumulation of viral products was not as efficient as that of the wild type in systemic leaves, which indicates that stem-loop A is dispensable for replication but signifies a role in systemic accumulation.

The gist of these results is that the 3′ UTR of BaMV RNA is involved in the initiation of minus-strand RNA synthesis, polyadenylation, and viral RNA long distance movement.

The *cis*-acting elements for BaMV plus-strand genomic RNA synthesis

To identify the promoter region for plus-strand genomic RNA synthesis, *in vitro* RdRp assay was used to examine short transcripts, 39-, 77-, and 173 nt, derived from the 3′ terminus of the minus-strand RNA for their ability to direct RNA synthesis (Cheng *et al*. 2001; Lin *et al*. 2005a). The 3′-terminal 77 nt RNA (Ba-77) was shown to be the most efficient RNA template with the minimal sequence required to form two stem-loops (Lin *et al*. 2005a). The enzymatic structural probing data supported the existence of the predicted structure with a large stable stem-loop and a small unstable stem-loop in Ba-77 (**Fig. 4**). Ba-77/Δ5 without the terminus UUUUC directed the RNA synthesis only 7% that of Ba-77 *in vitro*. Mutant with a deletion of 16 or 31 nt from the 3′-end but retaining the internal UUUUC repeating unit as the terminal UUUUC could partially preserve the template activity to about 60%. These results indicated that UUUUC sequence at the very 3′-end was important for plus-strand RNA synthesis (Lin *et al*. 2005a).

Moreover, mutations that alter the sequence at the large stem-loop significantly reduced the RNA synthesis *in vitro* and viral RNA accumulation *in vivo*, which suggests that the sequence of the stem is involved in regulating the BaMV plus-strand RNA synthesis. Besides the terminal UUUUC and the large stem-loop, the distance between these two regions could also play a role in regulating the efficiency of BaMV plus-strand RNA synthesis. Mutants that shorten distance between these two regions reduced the levels of plus-strand RNA synthesis *in vitro* and of viral RNA accumulation *in vivo* (Lin *et al*. 2005a).

All of these results suggest that the replicase complex could have at least 2 different RNA interacting domains: the one in the catalytic core requires the template containing the UUUUC terminal sequence and the other domain is the specificity determinant which recognizes the large stem-loop.

Ba-39 is a short template with a UUUUC terminal sequence that could directly fit in the catalytic core and initiate RNA synthesis but far less efficient. However, to be an effective template a large molecule such as Ba-77 requires both interactions. Therefore, shortening the distance between these 2 RNA moieties will interfere with the efficiency of RNA synthesis.

Overall, there are at least 3 *cis*-acting elements in the 3′-end of BaMV minus-strand RNA which are required to efficiently initiate viral RNA synthesis, the terminal UUUUC, the sequence of the large stem-loop, and the distance between these two regions.

THE *TRANS*-ACING FACTORS IN BaMV INFECTION CYCLES

We have been using two strategies to identify the *trans*-acting factors involved in BaMV infection cycle. The first aims for specific interaction with viral RNAs identified by electrophoretic mobility shift (EMSA), UV cross-linking, footprinting, and competition assays. The second aims to

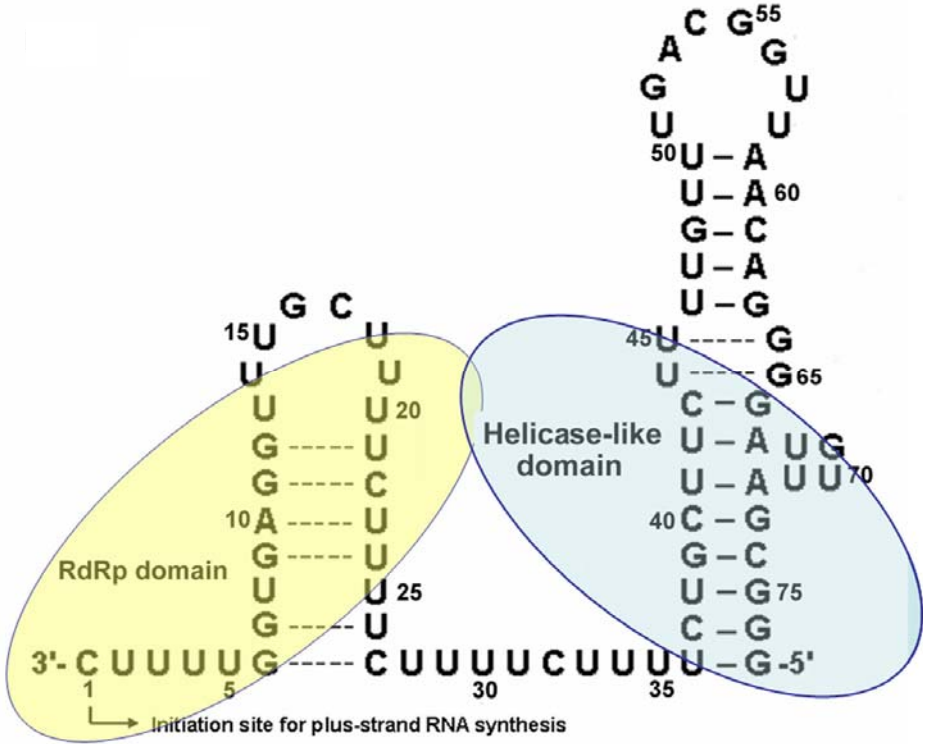

Fig. 4 The secondary structure of the 3′-terminal 77 nt of BaMV minus-strand RNA. The *cis*-acting elements required for efficient plus-strand genomic RNA synthesis studied *in vitro* and *in vivo* are indicated in reds and described in boxes. The initiation site of plus-strand RNA synthesis is also indicated. The RdRp domain shown in light yellow and the helicase-like domain shown in light blue with oval shape are indicated. (Adapted and modified from Tsai *et al.* 2006).

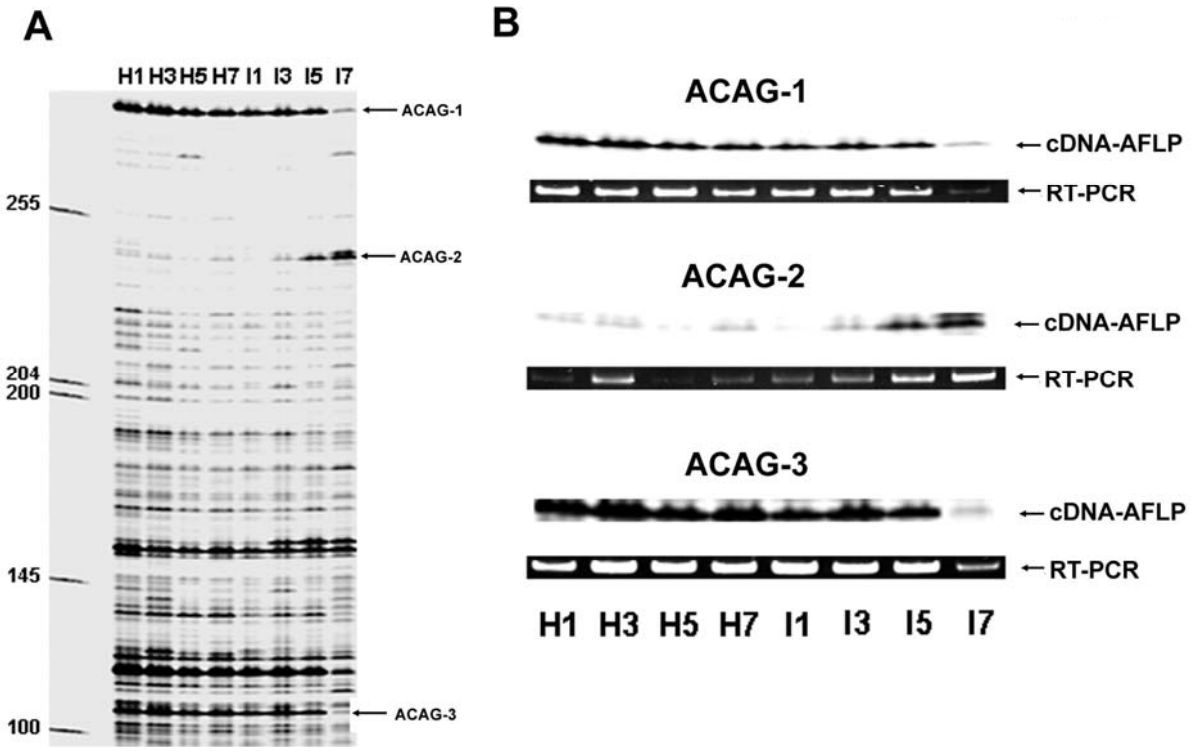

Fig. 5 The gene expression profile of *Nicotiana benthamiana* leaves during BaMV transfection. (**A**) Fluorescent cDNA-AFLP fragments were separated on 6.5%polyacrylamide urea denaturing gel and imaged with a fluorescent scanner. The size markers are indicated at the left of the gel. The arrows indicate the examples of three differential expressed fragments and were shown in (**B**). The mock and BaMV RNA inoculated samples are shown with H and I, respectively. The time (days) of the samples harvested post inoculation is shown above each lane. (**B**) The expression profile of the differential expressed cDNA fragments directly taken from (**A**) as indicated and confirmed with RT-PCR shown under the cDNA-AFLP.

detect up- or down-regulation of host genes during BaMV infection employing cDNA-amplified fragment length polymorphism (cDNA-AFLP) (Money *et al*. 1996) followed by a gene knock-down experiment of the gene of interest to examine the relationship between virus and the host gene.

The *trans*-acting factors derived from BaMV

The *Escherichia coli*-expressed RdRp domain and helicase-like domain derived from BaMV ORF 1 were used to demonstrate the specific binding to the 3′ UTR (Huang *et al*. 2001; Cheng *et al*. 2002; Chen *et al*. 2003). Results indicated that the RdRp domain binds to at least two independent RNA binding sites: the stem-loop D and the poly(A) tail (**Fig. 3**). Footprinting analysis revealed that RdRp could protect the sequences at the D domain containing the hexamer motif, part of the stem, and approximately 20 nt of the poly(A) tail adjacent to the 3′ UTR which matches to the region used to initiate minus-strand RNA synthesis (Huang *et al*. 2001; Cheng *et al*. 2002). UV cross-linking and competi-

tion experiments indicated that that the helicase-like domain could preferentially interact with the ABC domain (**Fig. 3**) (Chen *et al*. 2003). Besides, the helicase-like domain of BaMV replicase can also specifically interact with the large stem-loop of the promoter for plus-strand RNA synthesis done by UV-crosslinking and competition assays. Unfortunately, the RdRp domain showed a weak interaction in the assay. Since the helicase-like domain is connecting to the RdRp domain of the replicase, the hypothetical model of the localization of these two domains is proposed (**Fig. 4**). The helicase-like domain recognized the large stem-loop and help the RdRp domain to localize at the very 3′-end of minus-strand to initiate the plus-strand RNA synthesis.

The *trans*-acting factors derived from host plants

Besides the viral-encoded RdRp, host factors are required for the formation of the replicase complex of RNA viruses. In Qβ-infected bacteria, the replicase complex comprised not only viral RdRp but also translation elongation factors EF-Ts and -Tu and the ribosomal protein S1 to synthesize the plus-strand RNA (Blumenthal and Carmichael 1979; Blumenthal 1980). Moreover, a ribosome-associated protein, HF1, is required for the synthesis of the minus-strand RNA (Barrera *et al*. 1993). Translation factors also participate in viruses replicating in eukaryotic cells; for example, translation elongation factor 1a (EF1a) was claimed to be involved in some viruses including TMV, *West Nile virus* (WNV), *Vesicular stomatitis virus*, and TYMV (Joshi *et al*. 1986; Mans *et al*. 1991; Blackwell and Brinton 1997; Das *et al*. 1998; Dreher *et al*. 1999; Zeenko *et al*. 2002; Matsuda *et al*. 2004).

Using EMSA and UV cross-linking competition technique, host proteins p51 and p43 were demonstrated to bind specifically to the 3′ UTR of BaMV RNA. Results derived from LC/MS/MS indicated that p43 is chloroplast phosphoglycerate kinase (PGK) (Lin *et al*. 2007), a well known ATP-generating enzyme involved the glycolytic, gluconeogenic, and photosynthetic pathways (Banks *et al*. 1979; McHarg *et al*. 1999). The other protein p51 could be EF1a and negatively regulates the RdRp activity *in vitro* (Lin *et al*. 2007). EF1a has also been reported to bind the tRNA-like structure of BMV (Bastin and Hall 1976) and claimed to function in the negative-regulation of TYMV minus-strand RNA synthesis (Matsuda *et al*. 2004). On the contrary, EF1a interacts with the 3′-terminal stem-loop of WNV RNA and facilitates viral minus-strand RNA synthesis (Davis *et al*. 2007).

cDNA-AFLP, a highly sensitive and efficient technique for studying gene expression (Money *et al*. 1996); delivers reproducible results and has advantages over other differential display methods (Bachem *et al*. 1996; Ditt *et al*. 2001). Therefore, we adopted this technique to isolate the genes which are up- or down-regulated in *N. benthamiana* plants when infected with BaMV. The experiment uses the cDNA derived from mRNAs isolated from leaves 1, 3, 5, and 7 days post-BaMV inoculation in comparison with those derived from mock-inoculated leaves. To reduce false positive bands during the cDNA-AFLP experiments, a couple of different batches of cDNAs must be prepared independently and compared on the same gel. The cDNA fragments with up- or down regulated expression profile can be easily identified when they lined up together (**Fig. 5**). As shown in the figure that three fragments, ACAG-1, ACAG-2, and ACAG-3, were isolated, cloned, and sequenced. The identity of these fragments as ferredoxin-NADP reductase, unknown protein, and chloroplast carbonic anhydrase, respectively, were found using BLAST in Genebank. According to the sequence, RT-PCR was used to confirm the expression profile (**Fig. 5B**). Hundreds of these cDNA fragments with up- or down-regulated expression profiles during BaMV infection can be isolated by this technique.

To inspect the possible functions of those genes identified from UV-crosslinking or cDNA-AFLP with reference to BaMV infection, we used the virus-induced gene silencing (VIGS) (Ruiz *et al*. 1998) system to knockdown those genes and see if it affects the accumulation levels of viral products. *Tobacco rattle virus* (TRV) is the viral vector we used in VIGS system which has been shown to be more efficient than other silencing vectors (Ratcliff *et al*. 2001). TRV silencing system has been successfully in many plants including *Lycopersicum esculentum* (Liu *et al*. 2002) and *N. benthamiana* (Hiriart *et al*. 2003) to knockdown the homologous genes.

We have been using VIGS to knockdown the expression of chloroplast PGK in *N. benthamiana* plants (**Fig. 6**) and showed a reduced level of BaMV accumulation (**Fig. 7**). These results suggested that chloroplast PGK plays an important role in efficient BaMV accumulation in plant cells. It has been speculated that the replication complex of BaMV RNA is associated with chloroplast (unpublished data). Therefore, the interaction between the chloroplast PGK and the BaMV RNA seems to suggest that chloroplast PGK could assist the viral RNA in targeting it to the

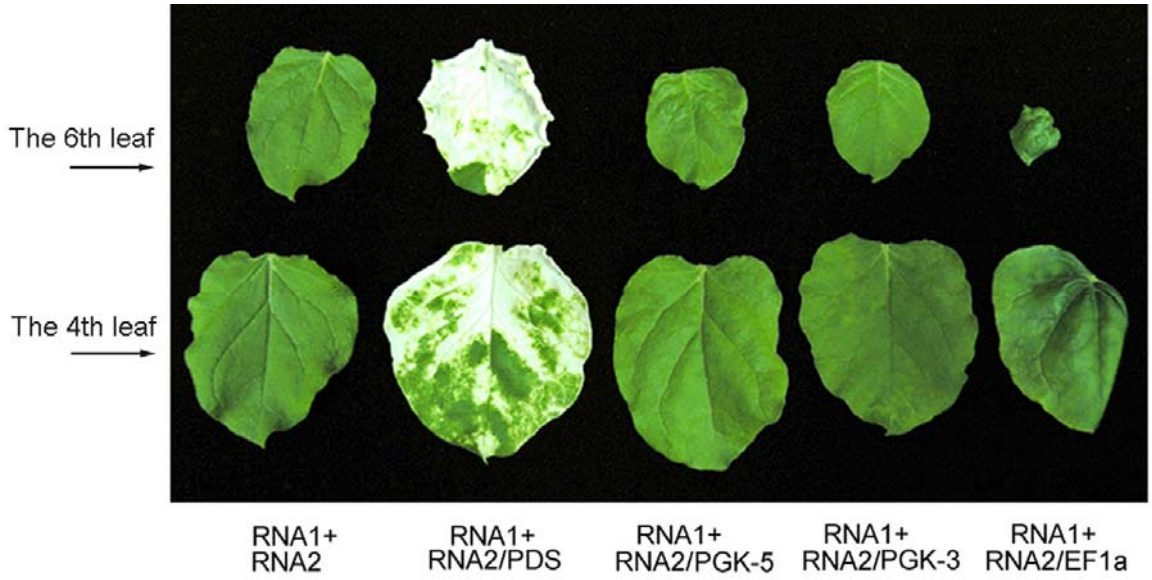

Fig. 6 The phenotypes of gene-knockdown plants. Arrows indicate the 6th (upper) and the 4th (lower) leaf above the infiltrated leaves of the gene-knockdown plants. The first from the left is the control plant showing no specific gene-knockdown. The second from the left is the phytoene desaturase (PDS) gene-knockdown plant showing a photobleaching effect. The next are two different chloroplast PGK gene-knockdown plants. (Adapted from Lin *et al.* 2007).

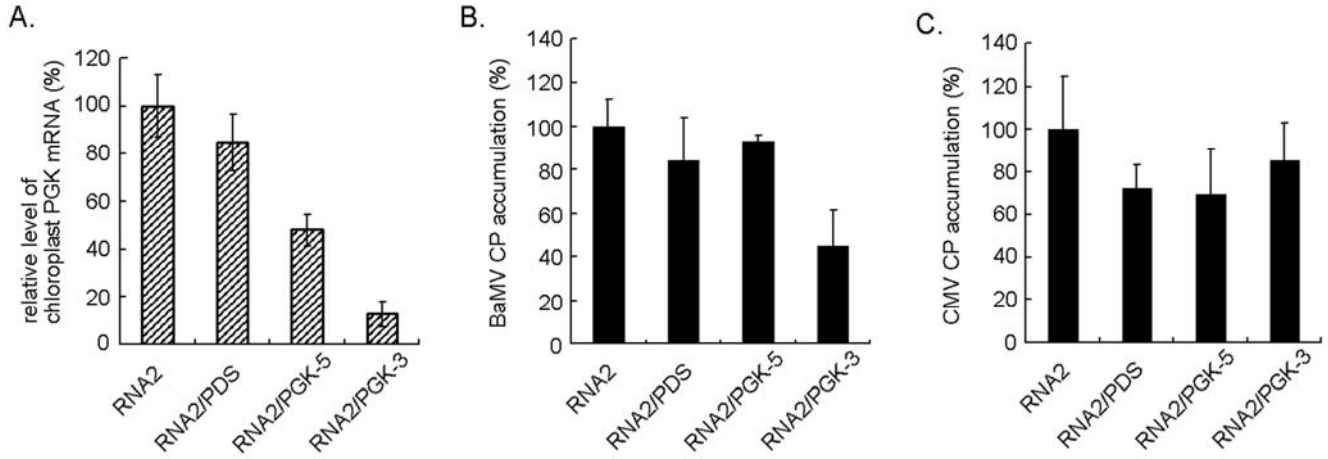

Fig. 7 Chloroplast phosphoglycerate kinase gene expression knock down experiment by virus-induced gene silencing system. The control (RNA2) and knock down plants (RNA2/PDS, RNA2/PGK-5, and RNA2/PGK-3) were challenged with 1 μg of the BaMV or CMV viral RNAs at the 4th leaf above the infiltrated leaves. Total RNAs and proteins were extracted from the virus inoculated leaves 48 hr post-inoculation. (**A**) The accumulation levels of chloroplast PGK mRNA were measured by real time PCR. The primer set for the amplification is targeted to the transit peptide region of the gene to measure the chloroplast-form mRNA. The accumulation levels of BaMV (**B**) and CMV (**C**) coat proteins were measured by Western blotting analysis with specific antiserums. All data were the averages (±standard deviations) of three independent experiments and normalized to that of pTRV2. (Adapted from Lin *et al.* 2007).

chloroplast membrane. Features of chloroplast PGK namely poly(A) binding and chloroplast localization will lead to further investigation into the possible functions of chloroplast PGK in viral RNA localization.

Knocking down the expression levels of a microtubule-associated protein (MAP) which was up-regulated at the 5th and 7th days post BaMV infection in *N. benthamiana* plants identified by cDNA-AFLP technique shows a reduced level of viral accumulation in inoculated leaves but not in protoplasts after transfected with BaMV RNA. These data suggest that the expression of MAP is required for efficient cell-to-cell movement but not for RNA replication (unpublished data).

CONCLUDING REMARKS

In combination of using a few techniques together such as MESA, UV-crosslinking, conventional chromatography, LC/MS/MS, we can identify a few specific *trans*-acting factors derived from host plants interacting with those *cis*-acting elements in the life cycle of BaMV. Using cDNA-AFLP technique, we can isolate hundreds of cDNA fragments derived from differential expressed genes during the infection of BaMV. The functional relationship of these *trans*-acting factors in the life cycle of BaMV infection can be revealed by VIGS techniques. Now, we have isolated a few hundred of differential expressed gene fragments from cDNA-AFLP. By using VIGS, we would expect to screen and identify the novel genes regulating the life cycle of BaMV infection positively or negatively.

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