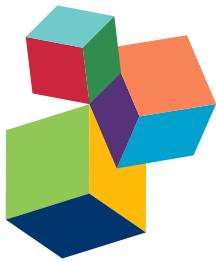


# **MOLECULAR BIOLOGY OF *BAMBOO MOSAIC VIRUS* – A TYPE MEMBER OF THE POTEXVIRUS GENUS**

**EDITED BY:** Yau-Heiu Hsu, Ching-Hsiu Tsai and Na-Sheng Lin

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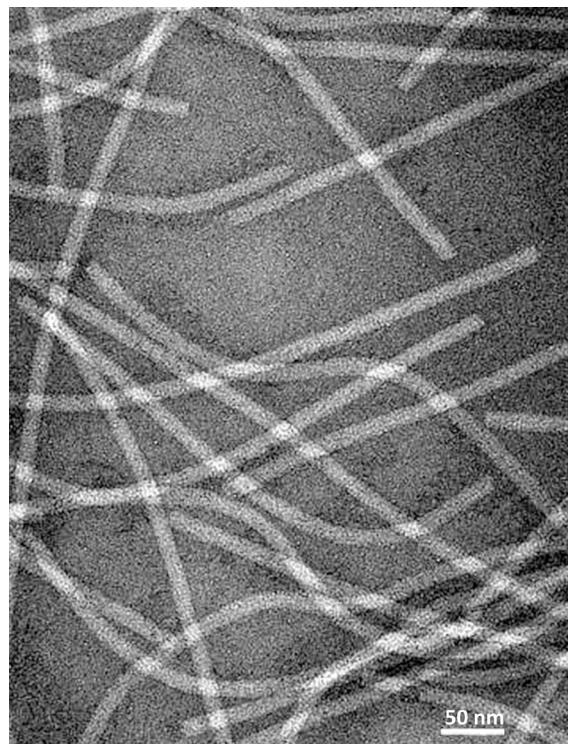
# MOLECULAR BIOLOGY OF *BAMBOO MOSAIC VIRUS* – A TYPE MEMBER OF THE POTEXVIRUS GENUS

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Electron micrograph of purified *Bamboo mosaic virus* in 0.05 M borate buffer, pH 8.0, stained with 2% uranyl acetate.

Image: Drs. Hsiung Wu and Chun-Chieh Chen.

The flexible filamentous plant viruses are responsible for more than half of all agricultural loss worldwide. Potexvirus is one of the two most important flexible filamentous plant viruses. *Bamboo mosaic virus* (BaMV), a single-stranded positive-sense RNA virus, is a member of the Potexvirus genus of Alphaflexiviridae. It can infect at least 12 species of bamboo, causing a

huge economic impact on the bamboo industry in Taiwan. The study of BaMV did not start extensively until the completion of the full-length sequencing of genomic RNA of BaMV and generation of the BaMV infectious cDNA clone in the early 1990s. Since then, BaMV has been extensively studied at the molecular, cellular and ecological level, covering both basic and applied researches, by a group of researchers in Taiwan.

In this eBook, the content comprises 6 reviews and 4 articles. Seven of them are involved in the infection of BaMV covering viral RNA replication, viral RNA trafficking, and the host factors. Two of them are related to the vector transmission and the ecology of BaMV. The last one is the application of using BaMV as a viral vector to produce vaccines in plants.

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# Editorial: Molecular Biology of *Bamboo mosaic Virus*—A Type Member of the Potexvirus Genus

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## Editorial on the Research Topic

### Molecular Biology of *Bamboo mosaic Virus*—A Type Member of the Potexvirus Genus

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Bamboo mosaic was first reported in Brazil in 1975, then in Taiwan, Australia, USA, India, China, etc. The causal agent, *Bamboo mosaic virus* (BaMV), is a member of the *Potexvirus* genus of *Flexiviridae*. BaMV can infect at least 12 species in 3 genera of bamboo. Symptoms include chlorotic mosaic on infected leaves and necrotic streaking on shoots and culms. With a high incidence of more than 90% infection in many bamboo plantations, the virus causes great economic loss, particularly in Taiwan, in terms of the quality and quantity of bamboo shoot production, a popular food.

However, study of BaMV did not start extensively until BaMV was identified as a member of the Potexvirus genus at the molecular level, in the early 1990s. Furthermore, the satellite RNA (satRNA) associated with BaMV (satBaMV) was found to be the only satRNA in Potexvirus. After completion of the full-length sequencing of genomic RNA of BaMV and generation of the BaMV infectious cDNA clone, study of BaMV stepped into a new dimension. Since then, BaMV has been extensively studied at the molecular level, covering different both basic and applied research, by a group of researchers in Taiwan. To date, more than 100 BaMV papers have been published in SCI international journals.

Like all potexviruses, BaMV has a flexuous morphology with a single-stranded positive-sense RNA genome. By investigating BaMV, the filamentous structure of flexible viruses was first determined at the near-atomic level by cryo-electron microscopy. This finding solved the mystery of how flexible virus particles maintain structural integrity as mechanical forces deform their structure. In addition, two types of subviral agents, defective RNA and satellite RNA, were found associated with BaMV in the field. Besides studying the viral RNA genomic structure and function, BaMV research has also focused on the virus-host interaction.

In this Research Topic, we summarize the BaMV research with 6 reviews and 4 research articles. To uncover the functional activity of replicase encoded by a positive-sense RNA virus, Meng and Lee describe the enzymatic activities associated with each of the functional domains of the BaMV-encoded replicase, including its unique capping mechanism, which may be conserved across the alphavirus superfamily (Meng and Lee). The authors also detail the interactions between replicase and the host proteins identified in *Nicotiana benthamiana*. Chen et al. cover the location of *cis*-acting elements in the viral RNA and their specific functions, including the recognition of the replicase complex for minus-strand, plus-strand and subgenomic RNA syntheses, viral RNA packaging, and viral movement. Moreover, the authors reveal the host factors that might be involved in delivering BaMV cargoes during intracellular movement,

including the delivery of viral RNA to the chloroplast for replication and viral RNA complex to move cell to cell (Cheng). Huang et al. describe the strategies used to identify the host proteins positively and negatively regulating the BaMV RNA replication and viral movements. One of the host proteins reported to be downregulated after BaMV infection is now revealed as carbonic anhydrase (Chen et al.). Nuclear-encoded chloroplast carbonic anhydrase may be involved in the re-initiation step of BaMV RNA replication.

In addition to host factors revealed in replication and movement, the study of BaMV-plant interactions explores a role for abscisic acid (ABA), a plant hormone, in the antiviral silencing pathway that could lead to interference in virus accumulation. Alazem and Lin describe how ABA affects the accumulation of BaMV and other viruses via the gene silencing pathway (Alazem and Lin). BaMV is unique in some isolates carrying satBaMVs, particularly the interfering satBaMV, with crucial roles in modulating BaMV replication and viral symptom development. Lin and Lin summarize the molecular mechanisms underlying the interaction of interfering satBaMV and BaMV (Lin and Lin).

Two papers are related to BaMV ecology. No insect vector for potexviruses has yet been uncovered. Chang et al. investigated the possibility of insect-mediated transmission of BaMV among bamboo clumps and found that two dipteran insects, *Gastrozona fasciventris* and *Atherigona orientalis*, could transmit BaMV to bamboo seedlings. The findings should help in managing BaMV infection by integrating the dipteran insect control. In addition, with decades of collections across a wide geographic area in Asia, Wang et al. have accumulated a sizable number of BaMV and satBaMV isolates for reconstruction of the BaMV and satBaMV phylogeny. The clustering results suggest that the Taiwan Strait

was a physical barrier to gene flow in the evolutionary history of both BaMV and satBaMV.

Finally, Chen et al. describe a BaMV-based vector system for peptide presentation. Chimeric BaMV virus expressing the epitope of Japanese encephalitis virus (JEV) could stimulate effective neutralizing antibodies against JEV infection in mice. This study demonstrates an alternative way to produce an effective vaccine candidate against JEV in plants by the BaMV-based vector system.

We thank our colleagues and friends, in total 27 authors, for their valuable contributions to this eBook and cohesive collaboration in the study of BaMV biology in the past 2 decades. We are also grateful to Dr. Anne Simon (University of Maryland), who encouraged us to initiate this Research Topic. We hope the knowledge we have gained from BaMV can serve as the groundwork and biotechnological application for Potexvirus research and RNA research as well.

## AUTHOR CONTRIBUTIONS

All authors listed have made substantial, direct and intellectual contribution to the work, and approved it for publication.

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# Function and Structural Organization of the Replication Protein of *Bamboo mosaic virus*

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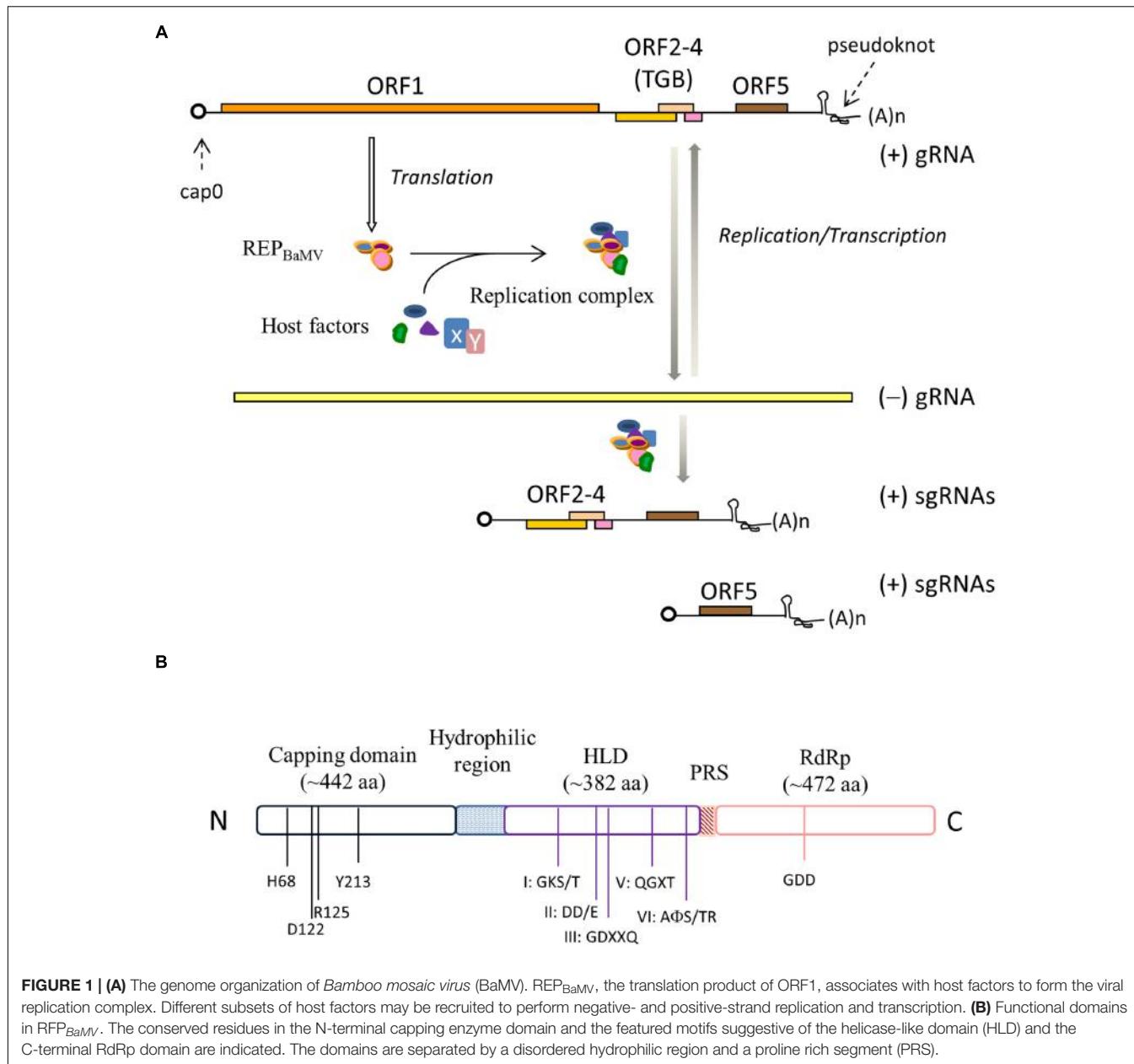
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The genus *Potexvirus* is one of the eight genera belonging to the family *Alphaflexiviridae* according to the Virus Taxonomy 2015 released by International Committee on Taxonomy of Viruses ([www.ictvonline.org/index.asp](http://www.ictvonline.org/index.asp)). Currently, the genus contains 35 known species including many agricultural important viruses, e.g., *Potato virus X* (PVX). Members of this genus are characterized by flexuous, filamentous virions of 13 nm in diameter and 470–580 nm in length. A potexvirus has a monopartite positive-strand RNA genome, encoding five open-reading frames (ORFs), with a cap structure at the 5' end and a poly(A) tail at the 3' end. Besides PVX, *Bamboo mosaic virus* (BaMV) is another potexvirus that has received intensive attention due to the wealth of knowledge on the molecular biology of the virus. In this review, we discuss the enzymatic activities associated with each of the functional domains of the BaMV replication protein, a 155-kDa polypeptide encoded by ORF1. The unique cap formation mechanism, which may be conserved across the alphavirus superfamily, is particularly addressed. The recently identified interactions between the replication protein and the plant host factors are also described.

**Keywords:** *Bamboo mosaic virus*, *Potexvirus*, RNA-dependent RNA polymerase, mRNA capping, virus-host interaction, positive-strand RNA virus, guanylyltransferase

## BaMV GENOME

*Bamboo mosaic virus* (BaMV) primarily infects members of the *Bambusoideae* in nature; nonetheless, it also replicates in *Nicotiana benthamiana*, which thereby has been used as the surrogate in laboratories. The RNA genome of BaMV contains 6366 nucleotides (nts) plus a 5' m<sup>7</sup>GpppG (cap0) structure and a 3' poly(A) tail (Figure 1A). It is functionally organized into a 94-nt 5' untranslated region (UTR), five ORFs, and a 142-nt 3' UTR (Lin et al., 1994). Two major subgenomic RNAs, co-terminal with the viral 3' UTR, would be produced once the virus starts to replicate in host cells. The first ORF encodes a 155-kDa non-structural protein (REP<sub>BaMV</sub>) that has been thought to be essential for replication/transcription of the viral genome and the formation of the 5' cap based on the presence of signature motifs of Sindbis virus-like methyltransferase (Rozanov et al., 1992), helicase (Habili and Symons, 1989), and RNA polymerase (Koonin and Dolja, 1993). As many positive strand RNA viruses, BaMV must encode its own enzymes for replication/transcription and 5' cap formation because it replicates only in the cytoplasm. ORF2, 3 and 4 are overlapped, often referred to as the triple gene block (TGB), and their translated proteins, TGBp1, TGBp2, and TGBp3, respectively, are indispensable for BaMV movement in plants (Lin et al., 2004, 2006). In-depth discussions about the functions of each of the TGB proteins of PVX in



**FIGURE 1 | (A)** The genome organization of *Bamboo mosaic virus* (BaMV). REP<sub>BaMV</sub>, the translation product of ORF1, associates with host factors to form the viral replication complex. Different subsets of host factors may be recruited to perform negative- and positive-strand replication and transcription. **(B)** Functional domains in REP<sub>BaMV</sub>. The conserved residues in the N-terminal capping enzyme domain and the featured motifs suggestive of the helicase-like domain (HLD) and the C-terminal RdRp domain are indicated. The domains are separated by a disordered hydrophilic region and a proline rich segment (PRS).

the intracellular trafficking and intercellular transport can be referred in a couple of recent reviews (Verchot-Lubicz et al., 2010; Park et al., 2014). ORF5 encodes the viral coat protein (CP) that is the only structural protein required for the assembly of BaMV virions. CP also exerts a critical function in the accumulation of BaMV RNAs in protoplasts (Lee et al., 2011). It is unclear whether CP protects BaMV RNAs from being destroyed by the host defense mechanisms or if it actually participates in the viral replication process. In addition, CP of potexvirus was reported to play a role in the virus movement. For instance, *White clover mosaic virus* needs CP to spread efficiently in plants (Forster et al., 1992), and PVX is defective in cell-to-cell movement if it carries a C-terminally truncated CP (Fedorkin et al., 2001). Occasionally, an 836-nt satellite RNA (satBaMV) is found in association with

BaMV in nature (Lin and Hsu, 1994). satBaMV contains one ORF that encodes a 20-kDa polypeptide (P20). P20 is not necessary for the replication of satBaMV; nonetheless, the accumulation rate of satBaMV in systemic leaves decreases in the absence of P20 (Lin et al., 1996).

## DOMAIN ORGANIZATION OF REP<sub>BaMV</sub>

There are apparently three functional domains in REP<sub>BaMV</sub> (**Figure 1B**), separated by a disordered hydrophilic region, from approximately amino acid residues 406–520, and a proline-rich segment (PRS), residues 895–910, according to a secondary structure prediction using the PHD algorithm (Rost et al.,

1994). The N-terminal one-third of REP<sub>BaMV</sub> shares a few dispersedly conserved residues with the putative Sindbis-like methyltransferase domains (Rozanov et al., 1992) of a variety of plant and animal alphavirus-like viruses such as *Brome mosaic virus* and *Semliki Forest virus* (Li et al., 2001a). Sequence comparison also revealed that the central domain contains several NTP-binding motifs of RNA helicase superfamily 1 (SF1) (Kadaré and Haenni, 1997) and the C-terminal domain contains featured motifs of RNA polymerases, e.g., the catalytic GDD motif (Koonin and Dolja, 1993). Since REP<sub>BaMV</sub> is barely discernible in BaMV-infected *N. benthamiana*, the enzymatic activity associated with each of the domains has been investigated using the domains expressed in heterologous hosts such as *Escherichia coli* and *Saccharomyces cerevisiae*.

## CAPPING ENZYME DOMAIN

The enzymatic activity of the N-terminal 442 amino acids of REP<sub>BaMV</sub> was successfully characterized by using the domain expressed in *S. cerevisiae* (Li et al., 2001a). The recombinant domain, strongly associated with the yeast membrane, could be radiolabeled by [ $\alpha$ -<sup>32</sup>P]GTP if S-adenosylmethionine (AdoMet) was provided in the reaction buffer. Alternatively, it could be radiolabeled by Ado[methyl-<sup>3</sup>H]Met when GTP was present. The radiolabeled moiety covalently linked to the domain was subsequently determined to be m<sup>7</sup>GMP. This [m<sup>7</sup>GMP-enzyme] adduct was thought to represent an intermediate in the pathway to form the 5' cap. In other words, this viral domain could be a guanylyltransferase (mRNA capping enzyme) except that it is covalently modified by m<sup>7</sup>GMP rather than GMP. In addition, this N-terminal domain of REP<sub>BaMV</sub> was found capable of catalyzing a methyl transfer reaction from AdoMet to GTP, leading to the formation of m<sup>7</sup>GTP, consistent with the prediction of its function as a methyltransferase. This viral domain was therefore proposed to possess an AdoMet-dependent guanylyltransferase activity, by which the methyl group of AdoMet is transferred to GTP, leading to m<sup>7</sup>GTP formation, and then the m<sup>7</sup>GMP moiety of m<sup>7</sup>GTP was transferred to an active-site residue to form the covalent [m<sup>7</sup>GMP-enzyme] intermediate. Analogous reactions have been observed also in other members of alphavirus-like superfamily including alphavirus (Ahola and Kääriäinen, 1995), *Brome mosaic virus* (Ahola and Ahlquist, 1999), *Semliki Forest virus* (Ahola et al., 1997), *Hepatitis E virus* (Magden et al., 2001) and *Tobacco mosaic virus* (TMV) (Merits et al., 1999), suggesting that this unique mRNA capping process is conserved throughout diverse members within the superfamily in spite of the fact that only limited amino acid identities (e.g., H68, D122, R125, and Y213 in REP<sub>BaMV</sub>) are conserved.

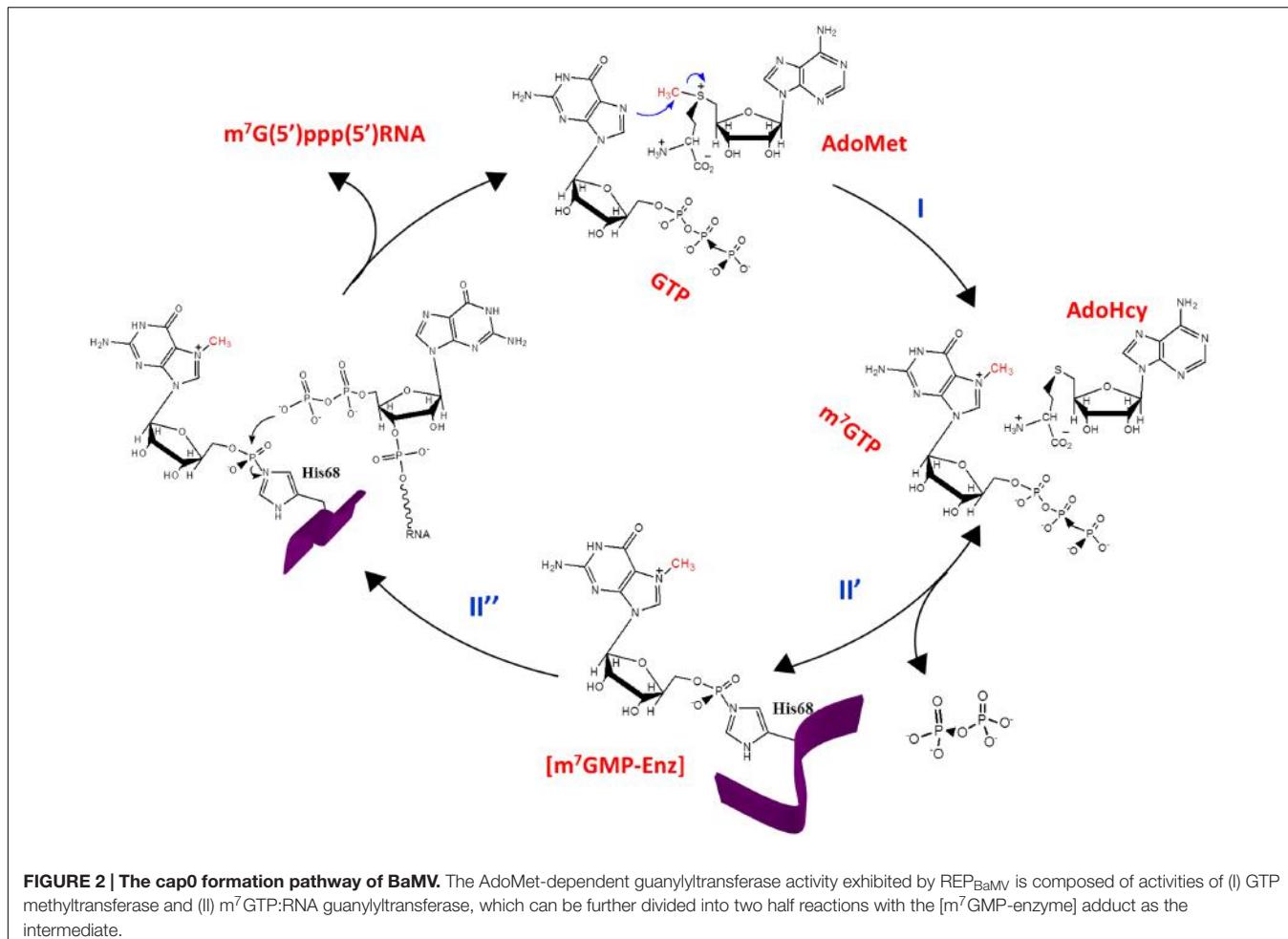
Site-directed mutagenesis indicated that H68, D122, R125, and Y213 are essential for the BaMV capping domain to form the covalent [m<sup>7</sup>GMP-enzyme] intermediate (Huang et al., 2004). Alanine substitution for each of the conserved residues, except H68, also disabled the domain to produce m<sup>7</sup>GTP (Huang et al., 2004). Intriguingly, H68A mutant increased m<sup>7</sup>GTP production by a factor of ~10, implying a special role of H68 in the pathway to form the covalent [m<sup>7</sup>GMP-enzyme] intermediate.

The H68A mutant was thus treated as the pseudo wild type to investigate the aromatic residues important for the formation of m<sup>7</sup>GTP (Hu et al., 2011). A number of aromatic residues, including Y126, F144, F161, Y192, Y203, Y213, and W222, were found critical for AdoMet recognition. Alanine substitution for these residues, except Y213, also reduced the binding affinity to GTP. Probably, the BaMV capping domain binds AdoMet and GTP in close proximity and many of these aromatic residues participate in the binding of the two substrates simultaneously. It is noteworthy that all the indicated aromatic residues are well conserved among the capping domains of potexviruses. The primary function of Y213 is to bind AdoMet. The inability to substitute phenylalanine for Y213 suggests that the hydroxyl group on Y213 provides an essential hydrogen bond to AdoMet. Presumably, Y213 locks AdoMet in a correct spatial position so that the methyl group from the electrophilic methylsulfonium of AdoMet can be transferred to the N7 of GTP.

Peptide mapping using alkaline hydroxylamine, which specifically cleaves the asparaginyl-glycyl bond (Bornstein and Balian, 1977), indicated that the m<sup>7</sup>GMP-linking residue of the BaMV capping domain is located within the region of residues 44–76 (Lin et al., 2012). The covalent [m<sup>7</sup>GMP-enzyme] intermediate was sensitive to 0.1 N HCl but tolerant of 0.1 N NaOH (Lin et al., 2012), suggesting that the link connecting the domain and m<sup>7</sup>GMP is a phosphoamide bond (Duclos et al., 1991). Amino acids with nucleophilic side chains including lysine, arginine, asparagine, glutamine, serine, threonine, tyrosine, and cysteine were used to replace His68 (Lin et al., 2012). All the mutants, except H68C, failed to form the covalent [m<sup>7</sup>GMP-enzyme] intermediate. H68C retained a detectable activity for the covalent intermediate formation despite at considerably lower extent. The bond connecting m<sup>7</sup>GMP and the H68C mutant enzyme was moderately stable in 0.1 N HCl and 0.1 N NaOH (Lin et al., 2012), a characteristic of a phosphocysteine bond (Duclos et al., 1991). The change of the nature of the bond connecting the enzyme and m<sup>7</sup>GMP and the result of peptide mapping lead to the conclusion that His68 acts as the nucleophile to attack the  $\alpha$ -phosphate of m<sup>7</sup>GTP, consequently leading to the formation of the covalent [m<sup>7</sup>GMP-enzyme] intermediate.

The catalytic step after formation of the [m<sup>7</sup>GMP-enzyme] intermediate was characterized by monitoring the transfer of <sup>32</sup>P-radiolabeled m<sup>7</sup>GMP of the covalent intermediate to various RNAs (Huang et al., 2005). A RNA transcript with 5'-terminal diphosphate is a prerequisite to receive m<sup>7</sup>GMP from the covalent intermediate, and RNA led by GDP is a better substrate than that led by ADP. The putative stem-loop structure in the 5' region of BaMV genome, nts 34–118, has a critical effect on the capping efficiency of the genomic RNA, suggesting that most of the cap formation events occur after the stem-loop sequence has been synthesized in nascent transcripts. This result also implies that the RNA polymerase domain and the capping domain of REP<sub>BaMV</sub> need to coordinate to some extent.

According to the data aforementioned and others, the cap formation pathway catalyzed by the capping domain of REP<sub>BaMV</sub> is delineated in **Figure 2**. (1) GTP and AdoMet bind to the capping domain of REP<sub>BaMV</sub> in proximity (Hu et al., 2011). The presence of AdoMet actually enhances the binding affinity of the



domain for GTP. (2) The precise disposition of GTP and AdoMet in the domain facilitates a nucleophilic attack of the N7 of GTP on the methyl group of AdoMet, leading to the production of m<sup>7</sup>GTP and S-adenosyl-L-homocysteine (AdoHcy). (3) The nitrogen atom (not determined whether N<sup>81</sup> or N<sup>82</sup>) of His68 functions as a nucleophile attacking the α-phosphate of m<sup>7</sup>GTP, under the assistance of Mg<sup>2+</sup>, to form the covalent [m<sup>7</sup>GMP-enzyme] intermediate (Lin et al., 2012). This step is reversible because excess pyrophosphate could drive the m<sup>7</sup>GMP moiety on the covalent intermediate backward to form m<sup>7</sup>GTP (Huang et al., 2004). (4) The 5'-terminal diphosphate of nascent RNA binds to the domain in proximity to the m<sup>7</sup>GMP moiety. The 5' β-phosphate of the RNA launches a nucleophilic attack on the phosphorus atom of m<sup>7</sup>GMP, leading to the break of the phosphohistidine bond. (5) Finally, the RNA with a 5' cap0 structure is released from the domain.

## HELICASE-LIKE DOMAIN (HLD)

The HLD of REP<sub>BaMV</sub> (residues 514–895) forms inclusion bodies when it is expressed in *E. coli*. This domain resumes soluble after denaturation and refolding processes. The purified HLD is able

to remove the γ phosphate from nucleoside triphosphates as well as RNA (Li et al., 2001b); in other words, it can be a nucleoside triphosphatase (NTPase) or RNA 5'-triphosphatase (5'-TPase), depending on the substrate. Both of these reactions required the presence of divalent Mg<sup>2+</sup> or Mn<sup>2+</sup> cations. Mutations at any of the signature motifs I, II, III, or VI of SF1 abrogate both types of activity (Han et al., 2007). Adenylyl-imidodiphosphate (AMPPNP), a non-hydrolyzable ATP analog, is a competitive inhibitor of the RNA 5'-TPase activity. The inhibition constant  $K_i$ (AMPPNP) was determined to be 93 μM, which is close to the  $K_m$  value of ATP (150 μM) for the NTPase activity (Han et al., 2007). The closeness between the values of  $K_i$ (AMPPNP) and  $K_m$ (ATP) and the simultaneous inactivation of both activities by mutations at the featured motifs of helicases suggest that a common catalytic site is used for the hydrolysis of both NTP and RNA. Nonetheless, the greater value of  $K_m$ (ATP) than  $K_m$ (RNA), which is about 2.5 fold, suggests that more active-site residues are involved in RNA binding. The peptidyl regions employed by the HLD to bind biotinylated RNA were mapped by the reversible formaldehyde crosslinking method followed by tandem mass spectrometry (Han et al., 2009). Five peptidyl regions were identified. Regions of residues 625–645 and 696–706 encompass the helicase motif I and II, respectively; while

regions of residues 585–610, 789–799, and 833–843 do not contain conserved sequences known to SF1. Compared with the well-characterized members in SF1, e.g., DNA helicase PcrA, the BaMV HLD seems to bind RNA using a different set of peptidyl regions. Mutagenesis of positively charged residues in these regions showed that some residues, e.g., K603 and R628, have a role in the virus movement (Han et al., 2009).

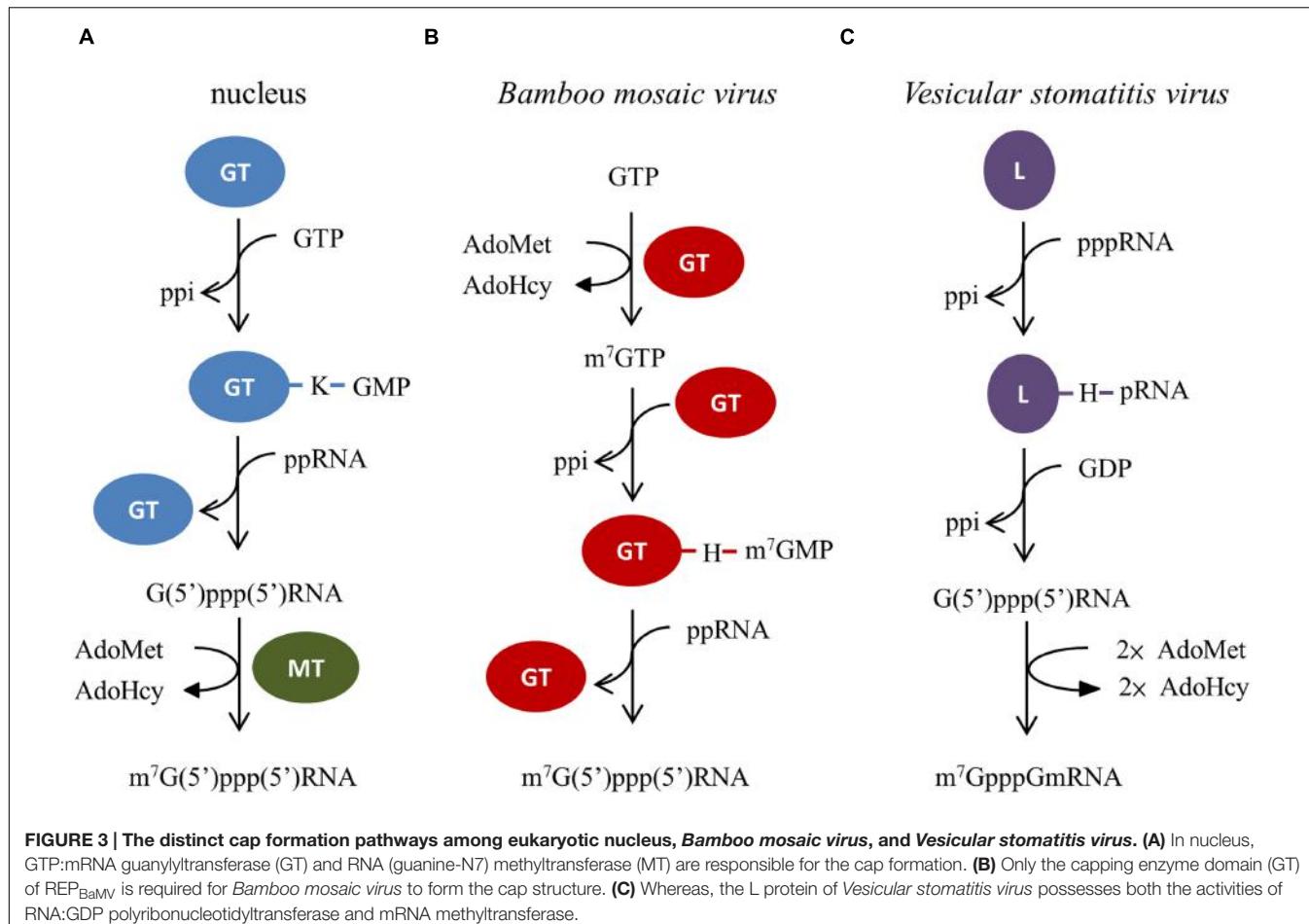
TGBp1 of BaMV is also a member of SF1. TGBp1 is capable of hydrolyzing NTPs but not RNA (Li et al., 2001b), implying that the RNA 5'-TPase activity embedded in the HLD of REP<sub>BaMV</sub> is not necessarily a property of all helicase proteins. The biological relevance of the RNA 5'-TPase activity was demonstrated in an *in vitro* assay, in which an RNA transcript would be capped at the 5' end by the capping domain of REP<sub>BaMV</sub> only if the RNA transcript had been pretreated with the HLD (Li et al., 2001b). Taken together, the first two domains of REP<sub>BaMV</sub> work in a concerted manner to complete the formation of the 5' cap on the nascent viral positive-strand RNAs. Besides participating in 5' cap formation, the HLD of REP<sub>BaMV</sub> has also been proposed to act as a *bona fide* helicase in the replication/transcription process of BaMV. Unfortunately, convincing evidence for duplex RNA-unwinding activity is still lacking even though a great deal of time and effort has been spent. To our knowledge, no helicase activity has been reported in the HLD of any other potexviruses. Perhaps, a more sophisticated assay is needed to discern this peculiar helicase activity. It is also possible that a host protein (other than a host helicase) may be recruited as an accessory subunit of the helicase to confer unwinding activity on the viral protein.

Yeast two-hybrid screen using a cDNA library prepared from BaMV-infected leaves of *N. benthamiana* identified a strong protein–protein interaction between the HLD of REP<sub>BaMV</sub> and the viral CP (Lee et al., 2011). Interacting with CP does not alter the *in vitro* enzymatic activity of the HLD. Mutations of A209G and N210S in CP, which diminish the CP-HLD interaction, were identified by a bacterial two-hybrid screen using a CP random mutant library generated by error-prone PCR (Lee et al., 2011). Mutant BaMV carrying A209G and/or N210S reproduces as efficiently as the wild type virus in *N. benthamiana* protoplasts (Lee et al., 2011). CP with the mutations retains a full activity for RNA binding, and the mutant virions exhibit similar morphologies as the wild type under transmission electron microscope (Lee et al., 2011). Nonetheless, the CP mutations do exert a profound effect on BaMV cell-to-cell movement in plants. With the A209G mutation, BaMV spreads much less effectively in leaves of *N. benthamiana* and *Chenopodium quinoa* (Lee et al., 2011). Notably, A209 of BaMV CP is well conserved among many potexviruses such as PVX and *Foxtail mosaic virus* (FoMV). A230G mutation in FoMV CP, analogous to BaMV A209G, also reduces the viral HLD-CP interaction and restricts the cell-to-cell movement of FoMV in *C. quinoa* (Lee et al., 2011). This finding suggests that the HLD-CP interaction is rather common in potexviruses; moreover, this interaction is relevant to the ability of the virus to move between cells. The critical role of the HLD-CP interaction in BaMV movement prompts us to suspect that REP<sub>BaMV</sub> is recruited into the viral movement complex, which is composed

of mainly the viral RNA, TGBps, and CP. More importantly, REP<sub>BaMV</sub> may pass through plasmodesmata along with the viral RNA. With this strategy, the viral RNA can be re-replicated immediately in the newly invaded cells so that the virus has a greater chance to defeat the silencing mechanism imposed by the hosts. Involvement of the replication protein in the viral movement complex has also been proposed in TMV based on the observation that TMV requires a significantly longer time for movement from primary inoculated cells to secondary cells than is required for movement from secondary to tertiary cells (Kawakami et al., 2004).

## A DISTINCT PATHWAY/MACHINERY FOR THE 5' CAP FORMATION

The 5' cap0, m<sup>7</sup>G(5')ppp(5')Np, in eukaryotic mRNAs is a basic structural unit required for mRNA export from the nucleus, prevention of mRNA degradation by 5'-exonucleases, and recognition by eIF4F complex to initiate the translation process (Furuichi and Shatkin, 2000; Shuman, 2001). Different pathways leading to the formation of the cap structure have been reported (Figure 3). Three consecutive enzymatic reactions are responsible for the cap formation in the nucleus. First, the γ-phosphate of a nascent mRNA is removed by RNA 5'-triphosphatase. The GMP moiety of GTP is then transferred to the 5' end of the 5'-diphosphorylated mRNA via a covalent enzyme-lysyl-GMP intermediate by GTP:mRNA guanylyltransferase. Finally, the guanine-N7 of G(5')ppp(5')Np cap is methylated by RNA (guanine-N7) methyltransferase to produce the cap0 structure (Mizumoto and Kaziro, 1987; Shuman, 1995). This canonical cap formation pathway also occurs in some DNA viruses, e.g., vaccinia virus (Shuman et al., 1980; Niles and Christen, 1993) and chlorella virus (Håkansson et al., 1997), and the double-stranded RNA reovirus (Luongo et al., 2000; Luongo, 2002). In the case of BaMV, the RNA 5'-triphosphatase activity embedded in the helicase-like domain of REP<sub>BaMV</sub> catalyzes the removal of γ-phosphate from the 5' end of nascent positive-strand RNA (Li et al., 2001b). The capping domain of REP<sub>BaMV</sub> exhibits an AdoMet-dependent mRNA guanylyltransferase activity, by which the methyl group of AdoMet is transferred to the N7 of GTP, and then the m<sup>7</sup>GMP moiety is transferred from the newly formed m<sup>7</sup>GTP to the 5' end of a 5'-diphosphorylated RNA via a covalent enzyme-histidyl-m<sup>7</sup>GMP intermediate (Li et al., 2001a,b; Huang et al., 2005; Lin et al., 2012). Plausibly, this type of cap formation pathway for BaMV also occurs across the alphavirus-like superfamily of human, animal, and plant-infection positive-strand RNA viruses. *Vesicular stomatitis virus* (VSV) performs another unconventional mRNA 5' cap formation pathway (Ogino and Banerjee, 2007; Ogino et al., 2010). Besides exhibiting a RNA-dependent RNA polymerase activity, the L protein of VSV has a RNA:GDP polyribonucleotidyltransferase activity that catalyzes the transfer of the 5'-monophosphorylated viral mRNA to GDP via an enzyme-histidyl-pRNA intermediate. Two methylation reactions at the capped RNA follow to form the cap1 structure by the viral methyltransferase activity.



**FIGURE 3 |** The distinct cap formation pathways among eukaryotic nucleus, *Bamboo mosaic virus*, and *Vesicular stomatitis virus*. **(A)** In nucleus, GTP:mRNA guanylyltransferase (GT) and RNA (guanine-N7) methyltransferase (MT) are responsible for the cap formation. **(B)** Only the capping enzyme domain (GT) of REP<sub>BaMV</sub> is required for *Bamboo mosaic virus* to form the cap structure. **(C)** Whereas, the L protein of *Vesicular stomatitis virus* possesses both the activities of RNA:GDP polyribonucleotidyltransferase and mRNA methyltransferase.

The BaMV enzymes performing the catalytic steps in the pathway are also unique from the viewpoint of protein structures. Apparently, the BaMV RNA 5'-triphosphatase activity has emerged from the helicase motif-containing domain. By contrast, the RNA 5'-triphosphatases of yeast and DNA viruses, e.g., vaccinia virus and baculovirus, belong to a metal-dependent phosphohydrolase family (Lima et al., 1999), while those of animals and plants are classified into a cysteine phosphatase superfamily (Changela et al., 2001). Moreover, the capping domain of REP<sub>BaMV</sub> does not share similarity in amino acid sequence with either GTP:mRNA guanylyltransferase or RNA (guanine-N7) methyltransferase of eukaryotic cells and DNA viruses. With the limited genome size, BaMV has evolved an efficient capping enzyme, with merely 442 amino acids, to accomplish the work of forming the 5' cap.

## RNA-DEPENDENT RNA POLYMERASE DOMAIN

The C-terminal domain of REP<sub>BaMV</sub> had been thought to be the key component of the viral replication complex, due to the presence of the hallmark signature of polymerase

S/TGX3TX3NS/TX22GDD (Koonin and Dolja, 1993). This domain (residues 893–1364), expressed in *E. coli* with a thioredoxin tag fused at the N terminus, exhibits an *in vitro* RNA polymerase activity, preferentially taking the 3'-terminal fragments of both positive and negative strands of BaMV as templates (Li et al., 1998). Mutational analysis confirmed the essential role of the GDD motif in the catalysis of polymerization reaction. Structure mapping based on selective RNA hydrolysis using a variety of ribonucleases and chemicals suggested that the 3' UTR of BaMV folds into four consecutive stem-loop domains (A–D), followed by a tertiary pseudoknot structure (Cheng and Tsai, 1999). The hexanucleotide ACC/UUAA, conserved in the 3' UTR of potexviruses, is situated in the apical loop of the D domain. A competition binding assay suggested that the *E. coli*-expressed BaMV polymerase domain binds independently to the D domain and the poly(A) tail (Huang et al., 2001). A footprinting assay further defined the D loop as the primary region protected by the polymerase domain of REP<sub>BaMV</sub> against chemical cleavages (Huang et al., 2001). Similarly, the 3'-terminal fragment (77 nts) of the BaMV negative-strand RNA was mapped to contain a 5' stem-loop, followed by a spacer and the 3'-CUUUU sequence (Lin et al., 2005). Reducing the number of uridylate in the 3'-CUUUU to less than three or changing the penultimate U to other nucleotides

is deleterious to BaMV accumulation in plants (Chen et al., 2010). UV-crosslinking and competition assay indicated that the *E. coli*-expressed BaMV polymerase domain also binds to the 3'-terminal fragment of the negative-strand RNA through a specific interaction particularly with the 5' stem-loop (Chen et al., 2010). In summary, the polymerase domain of REP<sub>BaMV</sub> recognizes the specific sequence and structural feature formed on the 3'-terminal region of both the positive and negative strands of BaMV, enabling the viral RNA replication to be initiated at the precise positions. Without these specific protein–RNA interactions, the replication of the viral RNAs would be incorrect or even impossible.

## SUBCELLULAR LOCALIZATION OF REP<sub>BaMV</sub>

In general, the replication complexes of plant positive-strand RNA viruses, which consist of the viral replication proteins, the viral genomic RNAs, and co-opted host factors, are embedded in membrane-enclosed micro-compartments derived from various cellular organelles (Novoa et al., 2005; Miller and Krijnse-Locker, 2008). Virus replication within the microenvironments should benefit the viral RNAs from being destroyed by the host defense mechanisms. For instances, *Brome mosaic virus* and *Red clover necrotic mosaic virus* recruit the membrane derived from endoplasmic reticulum to constitute their replication complexes (Noueiry and Ahlquist, 2003; Turner et al., 2004), while *Flock house virus* and *Tomato bushy stunt virus* employ the membrane of mitochondria and peroxisome, respectively, for replication complex assembly (Miller et al., 2001; McCartney et al., 2005). REP<sub>BaMV</sub> is also a membrane-associated protein. In fact, the membrane fraction P30 of BaMV-infected leaves, the pellet of cell extract after 30000 × g centrifugation, exhibits an *in vitro* BaMV RNA-dependent RNA polymerase activity; therefore, the P30 has been used in analysis of the *cis*-acting RNA elements required for the viral genome replication (Chen et al., 2003, 2005, 2010; Lin et al., 2005). To locate the subcellular organelle where BaMV replicates, a genetically modified BaMV positive-strand RNA that contains a phage MS2 CP-recognized sequence was inoculated into *N. benthamiana* leaves that had been infiltrated with *Agrobacterium tumefaciens* carrying the NLS-GFP-MS2 fusion protein-encoding gene (Cheng et al., 2013). The viral RNA was found located in chloroplasts according to the green fluorescent imaging of the infected cells under confocal microscope. Therefore, BaMV was proposed to replicate in chloroplasts although REP<sub>BaMV</sub> *per se* was invisible in the virus-infected leaves under microscope due to the low expression amount.

The chloroplast is a common target of a large number of plant viruses belonging to a variety of genera. Subcellular localization of the virus-encoded proteins in the chloroplast may constitute a basis for the viral pathogenesis or/and is critical for the viral propagation (Zhao et al., 2016). Besides BaMV, *Alternanthera mosaic virus* (AltMV) and PVX are two other potexviruses that have been demonstrated to be associated with

chloroplasts in their infection processes. The TGB3 of AltMV preferentially accumulates around the chloroplast membrane and disruption of TGB3 targeting to chloroplast impairs cell-to-cell movement of the virus (Lim et al., 2010). Furthermore, AltMV TGB3 strongly interacts with the photosystem II oxygen-evolving complex protein PsbO and this interaction correlates with chloroplast vesiculation and veinal necrosis caused by TGB3 over-expression (Jang et al., 2013). In the case of PVX, the viral CP interacts with the transit peptide of plastocyanin, a protein involved in photosynthesis, and silencing of plastocyanin prior to PVX infection reduces CP accumulation in chloroplasts and ameliorates symptom severity in host plants (Qiao et al., 2009).

## HOST PROTEINS ASSOCIATED WITH REP<sub>BaMV</sub>

A number of approaches have been used in the search for host proteins involved in regulation of the polymerase activity of REP<sub>BaMV</sub>. A biochemical protocol, basically involving steps of (1) UV-induced crosslinking of proteins in leaf cell extract to the <sup>32</sup>P-radiolabeled 3' UTR of BaMV, (2) nuclease digestion, and (3) radiolabeled protein identification using mass spectrometry, has identified several 3' UTR-interacting proteins including chloroplast phosphoglycerate kinase (PGK), cytosolic glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and heat shock protein 90 homolog (NbHsp90). PGK promotes BaMV accumulation presumably by facilitating transport of the viral genomic RNA to chloroplasts, the plausible replication site for BaMV (Lin et al., 2007; Cheng et al., 2013). GAPDH binds to the pseudoknot poly(A) tail of BaMV and reduces the replication efficiency of the viral negative-strand RNA probably through a competition with REP<sub>BaMV</sub> for RNA binding (Prasanth et al., 2011). NbHsp90 enhances BaMV replication presumably by either promoting the maturation of REP<sub>BaMV</sub> or bridging the interaction of REP<sub>BaMV</sub> with the viral RNA (Huang et al., 2012). The physical interaction between NbHsp90 and REP<sub>BaMV</sub> was actually confirmed by a yeast two-hybrid assay. Yeast two-hybrid screen was also used to search for host proteins interacting with the polymerase domain of REP<sub>BaMV</sub>. An uncharacterized host AdoMet-dependent methyltransferase (PNbMTS1) was thus isolated from the cDNA library prepared from *N. benthamiana* leaves (Cheng et al., 2009). PNbMTS1 exhibits an AdoMet-dependent inhibitory effect on BaMV CP accumulation in protoplasts. By contrast, *Tobacco rattle virus*-induced gene silencing of PNbMTS1 increased BaMV CP and genomic RNA in *N. benthamiana*. Both the membrane-targeting signal peptide and the AdoMet-binding motifs are essential for PNbMTS1 to suppress BaMV accumulation. Collectively, PNbMTS1 may have a role in the plant innate defense mechanism. Nonetheless, the target of PNbMTS1 relevant to the inhibition effect is still unknown.

Recently, we found that the expression of REP<sub>BaMV</sub> in *N. benthamiana* could be significantly enhanced if satBaMV was co-expressed. Probably, the positive-strand RNA of satBaMV might act as a template to facilitate the folding of REP<sub>BaMV</sub> or

prevent REP<sub>BaMV</sub> from being degraded by host proteases. Based on this finding, a proteomic approach was set up to find out the plant proteins differentially present in the REP<sub>BaMV</sub>-enriched P30 fraction (Lee et al., 2016). This approach includes steps of (1) transient expression of the hemagglutinin tag (HA)-fused REP<sub>BaMV</sub> and satBaMV, or satBaMV alone as the comparative control, in *N. benthamiana* by agroinfiltration, (2) preparation of the P30 fraction from the agroinfiltrated leaves, (3) protein solubilization using anionic detergent Sarkosyl, (4) protein precipitation using anti-HA antiserum, and (5) identification of the co-precipitated proteins by tandem mass spectrometry. Accordingly, dozens of host proteins were identified. To examine the role of the proteins in BaMV replication, each of the genes was transiently silenced in *N. benthamiana*. Those plants without apparent changes in phenotype were then challenged with a genetically modified BaMV that carries GFP as a reporter gene. Several potential host factors affecting BaMV replication were thus identified based on the effect of gene silencing on GFP expression. A cytoplasmic 5'→3' exoribonuclease (NbXRN4), a ripening-related protein, S-adenosylmethionine synthetase, and a respiratory burst oxidase homolog were found capable of promoting BaMV replication. By contrast, NADP<sup>+</sup>-dependent isocitrate dehydrogenase and MAP kinase phosphatase-like protein appeared to suppress BaMV replication. The relevance between the activity of NbXRN4 and BaMV replication was further investigated. In brief, NbXRN4 benefits BaMV replication, probably by removal of the uncapped genomic and subgenomic RNAs produced erroneously during the replication/transcription process.

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## PERSPECTIVE

Studies on replication-related proteins of plant RNA viruses have long been limited by inefficient protein expression and difficulty in protein purification. The catalytic characteristics of REP<sub>BaMV</sub> may thus not only apply to other members of the *Potexvirus* but also serve as references for those of other genera that also belong to the alphavirus-like superfamily. Nonetheless, the structural information at the atomic level regarding the functional domains of REP<sub>BaMV</sub> is still lacking, thanks mostly to the aggregation nature of these viral proteins. Methods that can overcome this obstacle are urgently needed. The search for host proteins, including those either boost or attenuate the enzymatic activity of REP<sub>BaMV</sub>, should be continued. More importantly, the mechanism underlying the function of host proteins should be elucidated so that the holistic and dynamic interplay between REP<sub>BaMV</sub> and its host can be understood.

## AUTHOR CONTRIBUTIONS

MM organized the contents of the text and wrote the manuscript. C-CL participated into the discussion about the text contents and approved the manuscript.

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# The Functional Roles of the *Cis*-acting Elements in *Bamboo mosaic virus* RNA Genome

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*Bamboo mosaic virus* (BaMV), which belongs to the genus *Potexvirus* in the family *Alphaflexiviridae*, has a single-stranded positive-sense RNA genome that is approximately 6400 nucleotides (nts) in length. Positive-sense RNA viruses can use genomic RNA as a template for translation and replication after entering a suitable host cell. Furthermore, such viral RNA is recognized by capsid protein for packaging and by viral movement protein(s) or the movement protein complex for cell-to-cell and systemic movement. Hence, viral RNA must contain signals for different functions to complete the viral infection cycle. In this review, we examine various *cis*-acting elements in the genome of BaMV. The highly structured 3' untranslated region (UTR) of the BaMV genomic RNA plays multiple roles in the BaMV infection cycle, including targeting chloroplasts for RNA replication, providing an initiation site for the synthesis of minus-strand RNA, signaling for polyadenylation, and directing viral long-distance movement. The nt at the extreme 3' end and the structure of the 3'-terminus of minus-strand RNA are involved in the initiation of plus-strand genomic RNA synthesis. Both these regions have been mapped and reported to interact with the viral-encoded RNA-dependent RNA polymerase. Moreover, the sequences upstream of open reading frames (ORFs) 2, 3, and 5 are involved in regulating subgenomic RNA synthesis. The *cis*-acting elements that were identified in BaMV RNA are discussed and compared with those of other potexviruses.

**Keywords:** positive-sense RNA virus, *Bamboo mosaic virus*, *cis*-acting elements, viral RNA replication, potexvirus

## INTRODUCTION

For a positive-sense RNA virus to establish a successful infection in a host, the viral RNA must house diverse *cis*-acting elements for minus-strand, plus-strand, and possibly subgenomic RNA syntheses (Dreher, 1999; Newburn and White, 2015). Furthermore, *cis*-acting elements could also be involved in cell-to-cell or systemic movement and encapsidation of viral RNA (Kwon et al., 2005; Lough et al., 2006; Cho et al., 2012; Rossmann, 2013). Studying the mechanisms of viral infections, localizing these *cis*-acting elements, and revealing their functional structures are critical steps in understanding viral infections at the molecular level. A few approaches were used to determine the minimum length and structures of viral *cis*-acting elements required for various functions. An *in vitro* replication assay is one of the most frequently used strategies to define the minimal requirement of *cis*-acting RNA elements for replication (Lin et al., 2005a,b; Osman et al., 2014). However, the difficulty involved in isolating a competent replicase preparation that can synthesize minus- or plus-strand RNAs, specifically with the *cis*-acting elements provided, limits

its use. The *cis*-acting elements discovered using this strategy were designated as promoters and are structured to specifically interact with the replicase. Structures of *cis*-acting elements have been computationally predicted and validated using enzymatic or chemical structural probing (Cheng and Tsai, 1999; Sun and Simon, 2006; McCormack et al., 2008). However, the structured *cis*-acting elements must be functionally verified by mutational analysis in either an *in vitro* replication assay or an *in vivo* infection assay.

*Bamboo mosaic virus* (BaMV) has a single-stranded positive-sense RNA genome that is approximately 6.4 kb in length with a 5'-cap structure and a 3' poly(A) tail. The genome contains five open reading frames (ORFs) (Figure 1). ORF1 encodes a replicase for viral RNA replication, ORF2 encodes a 28-kDa protein (a silencing suppressor) required for viral movement, ORF3 and ORF4 encode membrane-anchoring proteins required for virus movement, and ORF5 encodes a 25-kDa capsid protein for viral encapsidation, movement, and symptom development.

The *cis*-acting elements of BaMV RNA involved in viral RNA replication, intracellular trafficking, and movement have been extensively studied in the last two decades. This report comprehensively reviews these studies and discusses the common theme of the roles of these *cis*-acting elements that could be applied to other members of potexvirus including the *Potato virus X* (PVX), one of the top 10 plant viruses in molecular plant pathology (Scholthof et al., 2011), and even to certain animal viruses of *Alphaviruses*.

## VIRAL RNA INTRACELLULAR TRAFFICKING

When a positive-sense viral RNA enters a host cell, the host translation system is used to synthesize the viral proteins. The newly translated viral proteins target a specific membrane, usually an organelle-associated membrane, and modify the membrane suitable for viral RNA replication (Ahlquist et al., 2003; Laliberte and Sanfacon, 2010; Diaz et al., 2012; Nagy, 2016). The RNAs of *Tobacco mosaic virus* (Kawakami et al., 2004; Nishikiori et al., 2006), PVX (Bamunusinghe et al., 2009), *Tomato ringspot virus* (Han and Sanfacon, 2003), *Cowpea mosaic virus* (Carette et al., 2000), and *Tobacco etch virus* (Schaad et al., 1997) are transported to the endoplasmic reticulum membranes. The RNA of *Tomato bushy stunt virus* is transported to the peroxisomes (McCartney et al., 2005). The RNA of *Melon necrotic spot carmovirus* is associated with the mitochondria (Mochizuki et al., 2009). The RNAs of *Turnip yellow mosaic virus* (Prod'homme et al., 2003) and *Turnip mosaic virus* (Wei et al., 2010) are transported to the chloroplast membranes. These observations indicate that different viruses associate with distinct organelle membranes for replication (Laliberte and Sanfacon, 2010).

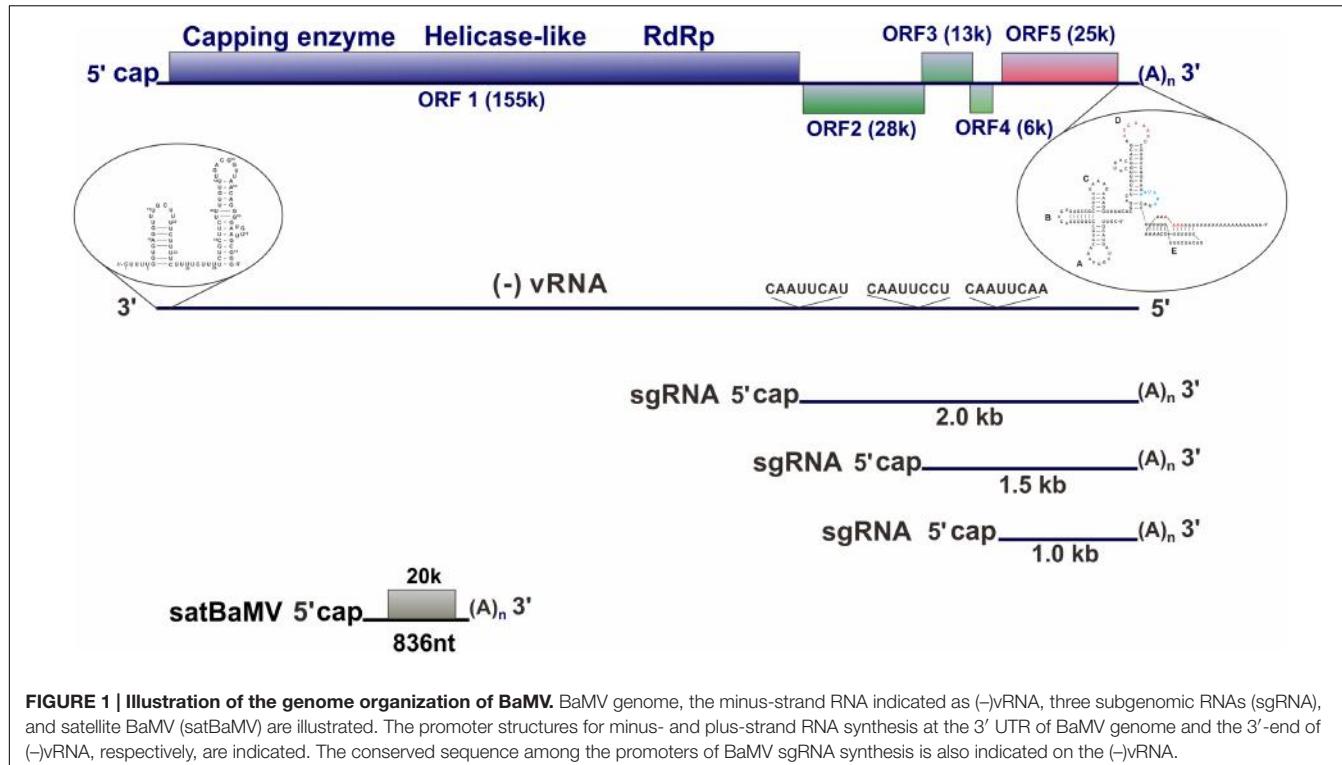
The mechanisms underlying the specific trafficking of viral RNA to targeted organelles for replication remain less known. In a recent study, BaMV was demonstrated to associate with chloroplasts for replication (Cheng et al., 2013). When the interaction between the 3' untranslated region (UTR) of BaMV

RNA (Figure 1) and host proteins in the replicase complex was studied, the involvement of elongation factor 1a (eEF1a) and chloroplast phosphoglycerate kinase (PGK) was revealed (Lin et al., 2007). A further study of the interactions indicated that a pseudoknot, including the poly(A) sequence at the extreme 3' end, is the target of PGK. *In vitro* and *in vivo* studies revealed that the interaction is required for efficient replication (Lin et al., 2007; Cheng et al., 2013). Notably, the chloroplast PGK can be replaced by a chimeric protein composed of cytoplasmic eEF1a and chloroplast RuBisCo small subunit (rbcS) (Cheng et al., 2013). These results suggest that nuclear-encoded chloroplast proteins, such as PGK and rbcS may serve to transport chloroplast-unrelated macromolecules into the chloroplasts by using their transit peptide. Once inside the host cell, the 3'-terminal pseudoknot and poly(A) sequence of BaMV RNA interact with PGK. The chloroplast PGK transit peptide facilitates entry into the chloroplast transport system. PGK and its associated macromolecules (BaMV RNA and possibly the translated replicase or the entire replicase complex) are transported into the chloroplasts (Cheng et al., 2013).

## MINUS-STRAND RNA SYNTHESIS

During initiation of minus-strand RNA synthesis, *cis*-acting elements located at the 3' end (usually in the 3' UTR) in most viruses play a critical role in recognition by the replicase complex. Typically, the 3'-terminal nucleotides (nt) or penultimate nt of non-poly(A)-tailed RNA viruses is used as the initiation site for minus-strand RNA synthesis (Dreher, 1999, 2009). However, the poly(A)-tailed RNA viruses have RNA genomes containing approximately 250 adenylates at the 3' end in the case of BaMV (Chen et al., 2005). Thus, *cis*-acting elements in the 3' UTR are far from the extreme 3' end of the initiation site if poly(A)-tailed viruses use a similar synthesis mechanism as described for non-poly(A)-tailed RNA viruses. Therefore, poly(A)-tailed RNA viruses might have a different strategy or use initiation sites that are close to the *cis*-acting elements. *In vitro* and *in vivo* studies of BaMV revealed that the extreme 5' end of minus-strand RNA contains stretch of uridine residues ranging from 1 to 15 nt, usually about 7–10 uridines (Cheng et al., 2002). These results indicate that the replicase complex assembles on the *cis*-acting elements in the 3' UTR, and that synthesis of minus-strand RNA initiates with uridylylate. The consequence of minus-strand RNA synthesis is that the subsequently synthesized plus-strand genomic RNA would have only a short stretch of adenylates at the extreme 3' end (<15 nts in length).

*Cis*-acting elements for minus-strand RNA synthesis, as mentioned previously, are usually situated in the 3' UTR and form secondary or tertiary structures, such as the stem-loops of *Alfalfa mosaic virus* (Houser-Scott et al., 1994; Reusken and Bol, 1996; Houser-Scott et al., 1997) and *Turnip crinkle virus* (Song and Simon, 1995) and the tRNA-like structures of *Brome mosaic virus*, *Tobacco mosaic virus*, and *Turnip yellow mosaic virus* (Kao and Sun, 1996; Osman and Buck, 1996; Deiman et al., 1997, 1998; Singh and Dreher, 1997).



**FIGURE 1 | Illustration of the genome organization of BaMV.** BaMV genome, the minus-strand RNA indicated as (-)vRNA, three subgenomic RNAs (sgRNA), and satellite BaMV (satBaMV) are illustrated. The promoter structures for minus- and plus-strand RNA synthesis at the 3' UTR of BaMV genome and the 3'-end of (-)vRNA, respectively, are indicated. The conserved sequence among the promoters of BaMV sgRNA synthesis is also indicated on the (-)vRNA.

The *cis*-acting elements for BaMV minus-strand RNA synthesis were also identified in the 3' UTR. The 3' UTR can be divided into three portions: the 5' part consisting of three stem-loops that form a cloverleaf-like structure, designated as the ABC domain; the middle part following the ABC domain, which is a major stem-loop with a bulge and an internal loop, designated as the D domain; and the 3' part of the UTR that forms the pseudoknot, described previously that interacts with eEF1a and PGK, covering a part of the poly(A) sequence adjoining the 3' UTR, designated as the E domain (Figure 2). Furthermore, results derived from ultraviolet (UV)-crosslinking and footprinting assays indicate that the polymerase and helicase-like domains of the replicase (ORF1 of BaMV) interact with the D and E domains and ABC domain of the 3' UTR, respectively. The potexviral conserved hexamer motif (ACXUAA) involved in the accumulation of virus was discovered in *Clover yellow mosaic virus* (White et al., 1992) and BaMV (Cheng and Tsai, 1999). This motif is located at the apical loop of the D domain in the 3' UTR of BaMV (Figure 2), and was protected from RNase digestion during interaction with the polymerase (Huang et al., 2001). The results of mutagenesis of this motif (ACCUAA in BaMV) indicate that the extreme 5' adenylate is a purine-specific nt, and the subsequent nt is by necessity a pyrimidine. The last three residues (UAA) are unalterable. The third nt affects viral accumulation less than the first two (Chiu et al., 2002).

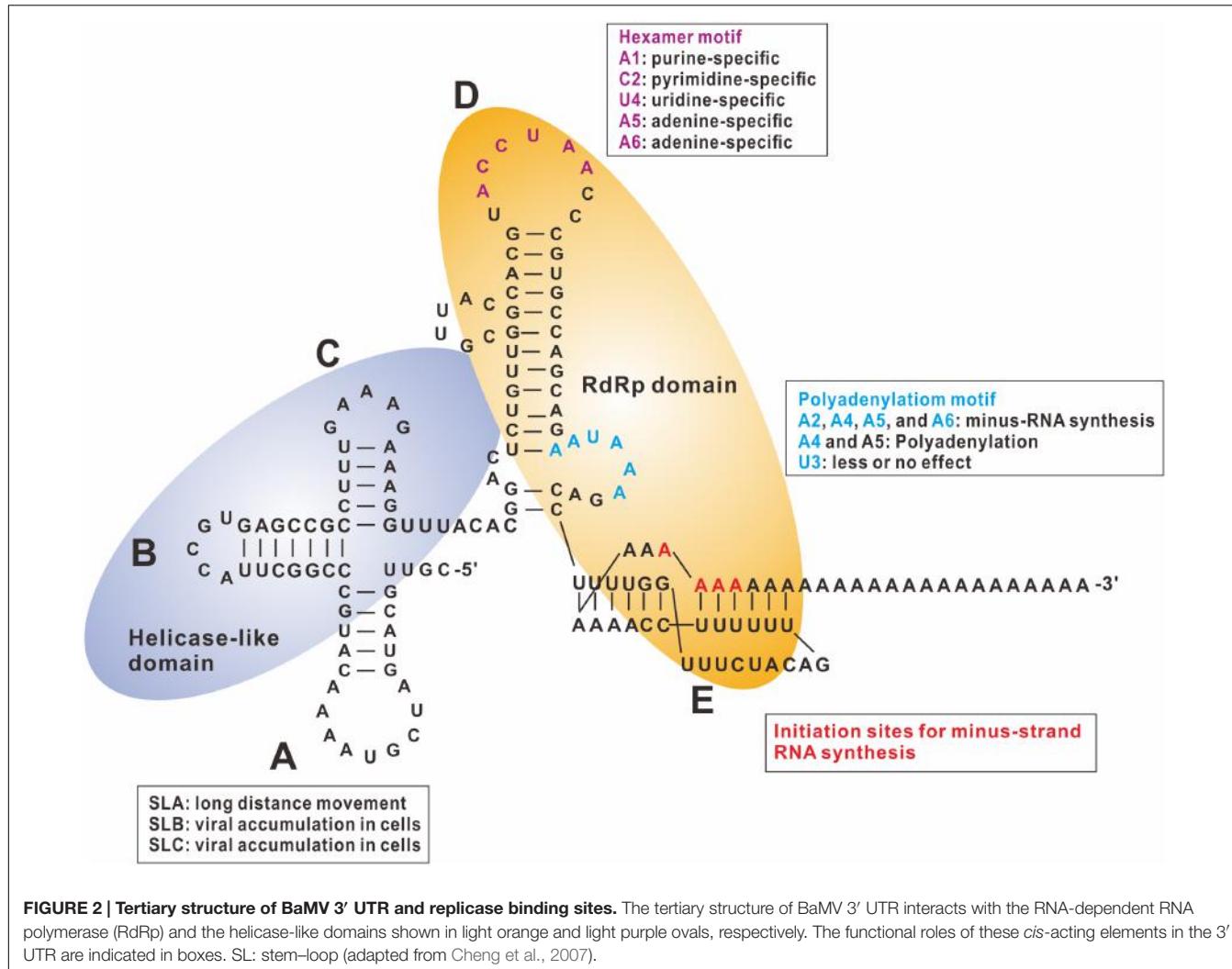
Maintaining the structures of D and E domains is critical for efficient viral RNA replication (Tsai et al., 1999). Mutations that disrupt the stems resulted in inefficient accumulation of viral RNAs. When compensatory mutations were introduced to re-form the stems, viral replication was restored. Furthermore,

retaining the pseudoknot structure of the E domain required 15 adenylates downstream (Cheng and Tsai, 1999). Viral full-length transcripts with <10 adenylates could not replicate sufficiently to be detected in the protoplasts. Transcripts with 15 adenylates at the 3' end could accumulate only up to 26% of the amount of wild-type transcripts with 25 adenylates (Tsai et al., 1999). These results suggest that the polymerase domain of the BaMV replicase interacts with stem-loop D specifically with the hexamer motif (ACCUAA) and the pseudoknot for initiation of minus-strand RNA synthesis. The initiation site for the minus-strand RNA synthesis in BaMV is not fixed at one position, but initiation starts at one of the 15 adenylates adjoining the 3' UTR (Cheng et al., 2002).

The stem-loops B and C of the ABC domain in the 3' UTR play a lesser, but significant, role in RNA replication (Chen et al., 2003). Accumulation of viral RNA in mutants with deleted stem-loop B or C was approximately 30% of wild type. Notably, accumulation of viral products of mutants with deleted stem-loop A did not differ significantly from that of wild type in protoplasts and inoculated leaves, but accumulation decreased dramatically in systemic leaves. These results suggest that stem-loop A is a *cis*-acting element for long-distance movement and does not play a role in RNA replication (Figure 2).

## PLUS-STRAND RNA SYNTHESIS

In an *in vitro* transcription assay, short transcripts of 39, 77, and 173 nts in length, corresponding to the 3' terminus of minus-strand RNAs, were used as templates to examine their ability



**FIGURE 2 | Tertiary structure of BaMV 3' UTR and replicase binding sites.** The tertiary structure of BaMV 3' UTR interacts with the RNA-dependent RNA polymerase (RdRp) and the helicase-like domains shown in light orange and light purple ovals, respectively. The functional roles of these *cis*-acting elements in the 3' UTR are indicated in boxes. SL: stem-loop (adapted from Cheng et al., 2007).

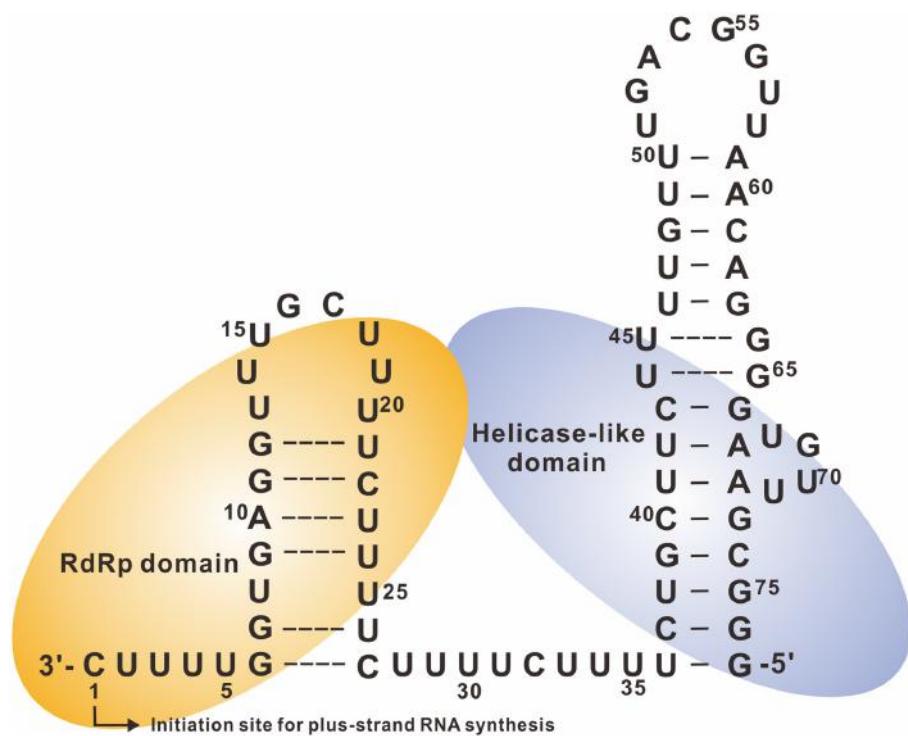
to direct RNA synthesis. The 3'-terminal 77-nt RNA, designated Ba-77, was the most efficient RNA template (**Figure 3**). It harbors two complete stem-loops confirmed by enzymatic structural probing and is required for plus-strand RNA synthesis (Lin et al., 2005a).

The terminal UUUUC pentamer is the most critical *cis*-acting element in BaMV for plus-strand RNA synthesis. Ba-77/Δ5, which lacks the terminal pentamer, exhibited only 7% template activity compared with that of Ba-77 *in vitro*. Ba-77 with an internal deletion of 16 or 31 nts (starting after the terminal pentamer) (**Figure 3**) and retaining the terminal UUUUC preserves up to 60% of the template activity of Ba-77 (Lin et al., 2005a). Furthermore, the sequence and structure of the large stem-loop at the extreme 5' end of Ba-77 are also vital for RNA synthesis. In mutants with altered sequences of the large stem-loop, RNA synthesis *in vitro* and viral RNA accumulation *in vivo* decreased significantly. Moreover, the sequence between the terminal pentamer initiation site and the large stem-loop may also play a significant role as mutants with shortening the sequence between

the terminal UUUUC and the stem-loop exhibited decreased accumulation of BaMV RNA *in vivo* and plus-strand RNA synthesis *in vitro* (Lin et al., 2005a).

At least three *cis*-acting elements at the 3' end of BaMV minus-strand RNA are required for efficient plus-strand genomic RNA synthesis, namely the 3'-terminal UUUUC pentamer motif, the sequence and structure of the large stem-loop, and the distance between these two regions. Accordingly, these *cis*-acting elements constitute the promoter for genomic RNA synthesis. The replicase contains two domains that interact with the promoter: the replicase catalytic center interacts with the terminal UUUUC sequence, and the specificity domain interacts with the large stem-loop (**Figure 3**) (Chen et al., 2010).

As mentioned previously, the extreme 5' end of minus-strand RNA has a short run of uridylates, copying from the poly(A) tail; therefore, the extreme 3' end of plus-strand RNA immediately after synthesis accordingly has a short run of adenylates (most frequently 7–10). However, to maintain the approximately 250 adenylates at the extreme 3' end of genomic RNA after synthesis, the *cis*-acting element AAUAAA in the 3' UTR plays a role



**FIGURE 3 | Secondary structure of the 3'-terminal 77 nts of BaMV minus-strand RNA and replicase binding sites.** The secondary structure of the *cis*-acting elements for genomic RNA synthesis interacts with the polymerase (RdRp) and helicase-like domains shown in light orange and light purple, respectively. The broken lines between base-pairing indicate that this region could be unstructured or has potentially unstable according to the probing results. The initiation site is numbered +1 for plus-strand RNA synthesis (adapted from Cheng et al., 2007).

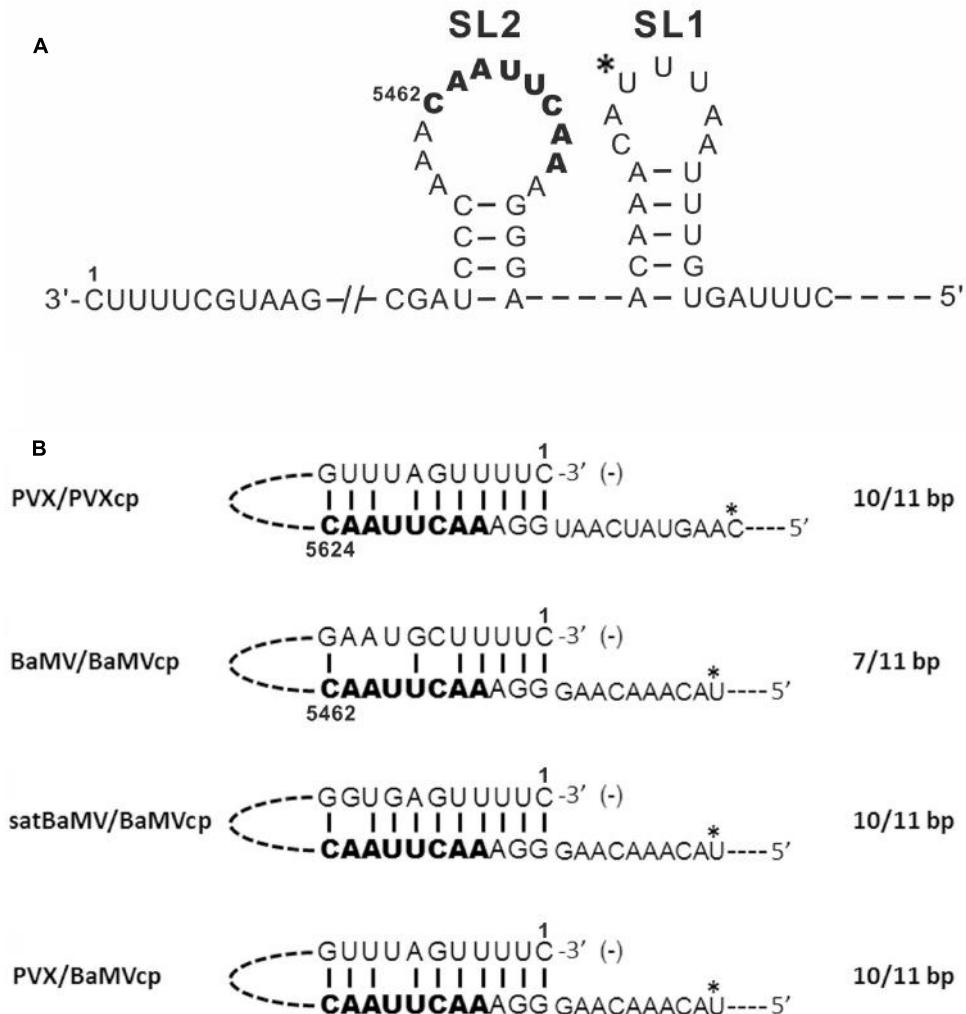
in polyadenylation (Chen et al., 2005). Interestingly, the *cis*-acting element for polyadenylation of BaMV RNA is identical to that of nuclear-encoded mRNAs. Whether BaMV uses an identical set of proteins as do nuclear-encoded host mRNAs for polyadenylation is an interesting question. A few observations oppose the aforementioned hypothesis on the use of identical proteins for polyadenylation. The host poly(A) polymerase is located mainly in the nucleus. Furthermore, the polyadenylation of mRNAs with poly(A) polymerase is independent of the recognition of the AAUAAA motif, whereas the polyadenylation of BaMV is associated with the AAUAAA motif (Chen et al., 2005).

## SUBGENOMIC RNA SYNTHESIS

The genomes of many positive-sense RNA viruses are multicistronic organizations that produce subgenomic RNAs (sgRNAs) to serve as messengers, allowing the translation of downstream ORFs (Sztuba-Solinska et al., 2011). A few strategies for synthesizing sgRNAs have been demonstrated, including internal initiation (Miller et al., 1985; Haasnoot et al., 2000), premature termination (White, 2002; Jiwan and White, 2011), and discontinuous synthesis (Sawicki and Sawicki, 1998; Pasternak et al., 2001). A short non-coding RNA derived from genomic RNA generated by host exonuclease is another

strategy to synthesize subgenomic RNA (Iwakawa et al., 2008). They commonly rely on *cis*-acting RNA elements to direct the viral-encoded RdRp to transcribe these RNAs (Newburn and White, 2015).

BaMV infection produces three sgRNAs with 3' coterminous. Two major sgRNAs of approximately 2 and 1 kb in length direct translation of ORF2 and ORF5, respectively (Lin et al., 1992). The other sgRNA, responsible for the translation of ORF3 and ORF4, is 1.5 kb in length accumulates in infected cells at a very low level. The satellite RNA of BaMV (satBaMV) was previously designed to be an expression cassette for examining *cis*-acting elements required for sgRNA synthesis (Lee et al., 2000). A cDNA covering the putative promoter region of BaMV sgRNA (SGP) was inserted into this cassette and resulted in sgRNA promoter-directed RNA synthesis in infected cells when coinoculated with BaMV. The *cis*-acting element of the SGP for synthesis of the 1-kb sgRNA covers the region between nt -91 to +52 (the transcription start site is designated as +1). Further analysis indicated that the SGP can be split into four elements: the core (nt -30 to +16), two upstream enhancers (nt -59 to -31 and -91 to -60), and a downstream enhancer (nt +17 to +52). The core sequence is the minimum region required for 1-kb sgRNA synthesis, which folds into two stem-loops, stem-loop (SL)1 and SL2, in minus strand (Figure 4A). Maintaining the integrity of SL2 structure and the conserved octamer motif (3'-CAAUUCAA-5') in the loop are essential for 1-kb sgRNA



**FIGURE 4 | Putative long-distance RNA–RNA interactions for subgenomic RNA synthesis. (A)** The predicted structure of the core sequence for BaMV 1-kb sgRNA synthesis (adapted from Lee et al., 2000). **(B)** Putative interaction of the 3'-end sequence of PVX, BaMV, or satBaMV minus-strand RNA with the conserved octamer motif upstream of the BaMV or PVX coat protein gene are indicated. The conserved octamer motif is indicated in bold font. The subgenomic RNA transcription start site is indicated using an asterisk. The predicted complementary base pairing is denoted.

synthesis. Furthermore, the *cis*-acting elements of SGP for 2-kb sgRNA synthesis are located at nt  $-119$  to  $+11$  (the transcription start site of the 2-kb sgRNA is designated as  $+1$ ). The minus-strand SGP sequence for 2-kb sgRNA synthesis was predicted to have similar stem-loops to those of the 1-kb SGP. The conserved octamer motif ( $3'$ -CAAUUCAU- $5'$ ) is also located in the loop of SL2 (Lee et al., 2000). Furthermore, the expected octamer motif ( $3'$ -CAAUUCCU- $5'$ ) for BaMV 1.5-kb sgRNA is located 12-nt upstream of the transcription start site.

Compared with the putative SGPs of ORF2 and ORF5 of potexviruses, the octamer motif is highly conserved (Kim and Hemenway, 1997; Lee et al., 1998). The long-distance RNA–RNA interaction between the conserved octamer motif and the 3'-terminal sequence of minus-strand genomic RNA was demonstrated to be required for transcription of PVX sgRNAs (Figure 4B) (Kim and Hemenway, 1999). As in an

ortholog, a similar interaction was revealed in BaMV with shorter complementary pairing than those in PVX (Figure 4B). Although BaMV SGPs were inserted into the satBaMV cassette without a BaMV minus-strand 3'-terminal sequence, the octamer motif could also interact with the 3'-terminal sequence of minus-strand satBaMV RNA (Figure 4B) (Lee et al., 2000). Redundant SGPs in a PVX-based expression vector were found to lead to genetic instability. The heterologous SGP from BaMV used in the PVX vector improves its stability for long-term production of proteins (Dickmeis et al., 2014). Complementarity between the octamer motif from the BaMV SGP and the 3'-terminal sequence of the minus-strand genomic RNA is required for transcription of sgRNA synthesis (Figure 4B). The long-distance RNA–RNA interaction between the 3'-terminal sequence and the conserved octamer motif of the SGPs observed in PVX and BaMV favors the internal initiation mode of sgRNA synthesis.

## CONCLUDING REMARKS AND FUTURE PROSPECTIVES

For accomplishing an efficient infection by a positive-sense RNA virus, the viral genome consists of various *cis*-acting elements for intracellular trafficking to organellar membranous target sites, minus-strand RNA synthesis, plus-strand genomic RNA synthesis, subgenomic RNA synthesis, viral movements, and viral encapsidation. In this review, we summarize studies of most of the *cis*-acting elements identified in the BaMV genome, except for those involved in viral movement and viral encapsidation. The signal for BaMV genomic RNA encapsidation is very likely in the 5' UTR, similar to those identified in PVX (Kwon et al., 2005; Karpova et al., 2006; Petrova et al., 2013, 2015). The structural elements and the functional roles for

the encapsidation of BaMV RNA will be revealed in the near future.

## AUTHOR CONTRIBUTIONS

I-HC wrote the section on the plus-strand RNA synthesis. Y-WH wrote the section of subgenomic RNA synthesis. C-HT wrote the rest part of the manuscript.

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# Host Factors in the Infection Cycle of Bamboo mosaic virus

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To complete the infection cycle efficiently, the virus must hijack the host systems in order to benefit for all the steps and has to face all the defense mechanisms from the host. This review involves a discussion of how these positive and negative factors regulate the viral RNA accumulation identified for the *Bamboo mosaic virus* (BaMV), a single-stranded RNA virus. The genome of BaMV is approximately 6.4 kb in length, encoding five functional polypeptides. To reveal the host factors involved in the infection cycle of BaMV, a few different approaches were taken to screen the candidates. One of the approaches is isolating the viral replicase-associated proteins by co-immunoprecipitation with the transiently expressed tagged viral replicase in plants. Another approach is using the cDNA-amplified fragment length polymorphism technique to screen the differentially expressed genes derived from *N. benthamiana* plants after infection. The candidates are examined by knocking down the expression in plants using the *Tobacco rattle virus*-based virus-induced gene silencing technique following BaMV inoculation. The positive or negative regulators could be described as reducing or enhancing the accumulation of BaMV in plants when the expression levels of these proteins are knocked down. The possible roles of these host factors acting on the accumulation of BaMV will be discussed.

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## INTRODUCTION

When a positive-sense RNA virus infects a host cell, it needs to produce its progeny and move it to the neighboring cells efficiently. In general, the entire infection cycle starts at the viral RNA entry, using the host translation system to produce the viral-specific replicase, transition the viral template from translation status to replication status, targeting the specific organelle for replication, rearrangement of the cellular membrane, recruitment of ancillary proteins to the replication site, viral RNA replication to synthesize the minus- and plus-strand RNAs, subgenomic RNA synthesis in some species, and finally, the viral-encoded movement proteins (MPs) and coat proteins accumulated for cell-to-cell movement and encapsidation, respectively. Some of the viral-encoded proteins that evolved not only fulfilled a specific role apart from amplification, but also performed counter-defense functions such as silencing suppressors against the virus-induced gene silencing system (Qu and Morris, 2005) or preventing the spread of the gene silencing signal (Voinnet et al., 2016).

*Bamboo mosaic virus* (BaMV) is a positive-sense, single-stranded RNA virus, belonging to the genus *Potexvirus* of the family *Alphaflexiviridae*. The genome of BaMV is approximately 6.4 kb in length with a 5' m<sup>7</sup>GpppG structure and a 3'-end poly(A) tail and contains five open reading frames (ORFs) (Lin et al., 1994). The 3' untranslated region (UTR) was demonstrated to form a complexed

structure (including a cloverleaf-like structure, a major stem-loop, and a pseudoknot) and acted as a *cis*-acting element for minus-strand RNA synthesis, polyadenylation, an intracellular trafficking signal, and long distance movement (Cheng and Tsai, 1999; Tsai et al., 1999; Chen et al., 2005; Lin et al., 2005; Cheng et al., 2013). ORF 1 encodes replicase, including a capping enzyme domain (Li et al., 2001a; Huang et al., 2004), a helicase-like domain (Li et al., 2001b), and an RNA-dependent RNA polymerase domain (Li et al., 1998). ORFs 2–4 overlapping genes termed “triple-gene-block” (TGB) encode the MPs (TGBp1, TGBp2, and TGBp3) involved in virus movement (Lin et al., 1994). ORF 5 encodes the capsid protein (CP) for virus encapsidation, movement, and symptom development (Lin et al., 1994; Lan et al., 2010; Lee et al., 2011; Hung et al., 2014). Furthermore, a satellite RNA (satBaMV) was identified to associate with BaMV and could be amplified by BaMV replicase (Lin and Hsu, 1994; Lin et al., 2006). The tertiary structures of the 5' and 3' UTRs of satBaMV were revealed to be similar to those of BaMV (Huang et al., 2009; Chen et al., 2010).

As mentioned previously, the positive-sense RNA virus has to establish an efficient replication after entry into a host cell; the host factors are usually required to join and form a multi-functional replication complex (Ahlquist et al., 2003; Nagy and Pogany, 2008; Huang et al., 2012a). The replicase complex isolated from Q $\beta$ -infected cells is composed of bacterial proteins, elongation factors EF-Tu and -Ts and ribosomal protein S1, and Q $\beta$  RdRp for plus-strand RNA synthesis (Blumenthal and Carmichael, 1979; Blumenthal, 1980). Additional bacteria protein HF1, a ribosome-associated protein, is required for the complex to synthesize the minus-strand RNA (Barrera et al., 1993). The eukaryotic translational elongation factor 1a (eEF1a) was revealed to be part of the replicase complex in tobamoviruses, tymoviruses, potyviruses, and tobusviruses (Joshi et al., 1986; Mans et al., 1991; Dreher et al., 1999; Nishikiori et al., 2006; Yamaji et al., 2006; Thivierge et al., 2008; Li et al., 2010; Luan et al., 2016).

A few strategies were used to identify the host factors involved in virus infection cycles. By screening the host cDNA library constructed in yeast with the two-hybrid technique, one can discover the specific host factor that interacted with the viral-encoded target protein, such as the replicase, MPs, or CP (Ren et al., 2000; Nagy, 2008; Schoelz et al., 2011). The virus-encoded proteins can also be used as a ligand to co-immunoprecipitation the possible candidates for interaction with the host (DeBlasio et al., 2015, 2016). In the UV cross-linking competition technique, host proteins could be identified as interacting with the viral RNA, such as the 5' or 3' UTRs (Lin et al., 2007; Huang et al., 2012b; Hyodo et al., 2014). The identities of the interacted candidates derived from co-immunoprecipitation or UV cross-linking techniques could be revealed by LC/MS/MS. The cDNA-amplified length polymorphism (AFLP), a highly sensitive and efficient technique used for studying gene expression (Money et al., 1996) and demonstrated to deliver reproducible results (Bachem et al., 1996; Ditt et al., 2001), was used to screen the host's differentially expressed genes in a post-virus infection (Cheng et al., 2010). The up- and downregulated cDNA fragments could be easily visualized and compared when run

in parallel on the gel. These differentially expressed cDNA fragments could be straightforwardly isolated, amplified, cloned, and sequenced.

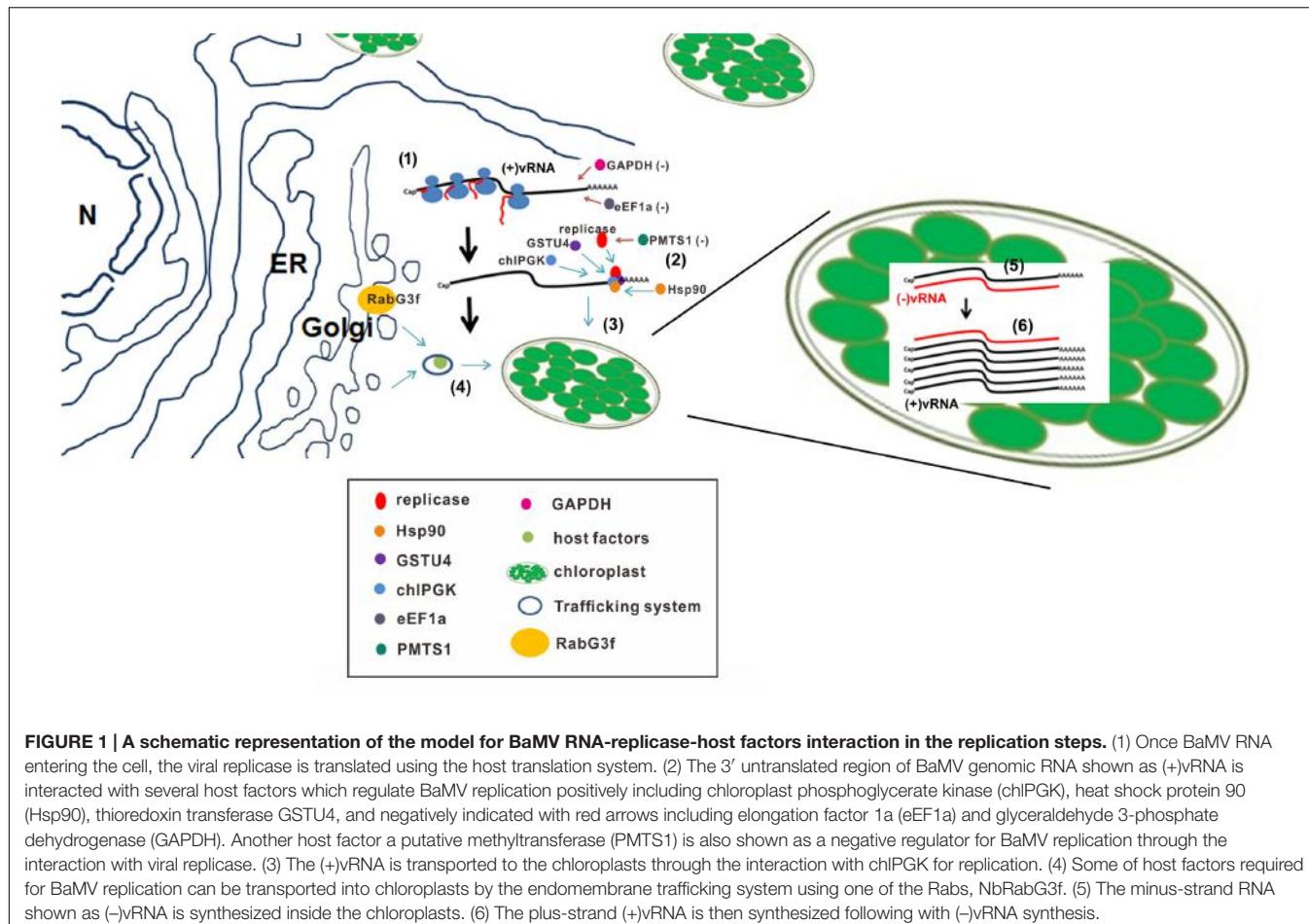
To reveal the relationship of the interacting host proteins with viral proteins or RNAs and the differentially expressed proteins in a post-virus infection, the *Tobacco rattle virus* (TRV)-based virus-induced gene silencing (VIGS) technique (Ruiz et al., 1998; Ratcliff et al., 2001) could be used to knock down the expressions and examine their effect on virus accumulation (Cheng et al., 2010). The results derived from the specific gene knockdown experiment (i.e., a loss of function) can be further confirmed by the complementary results derived from a transient expression of the same gene (i.e., a gain of function). These results would reveal whether the specific gene is playing an assistant or defense role in the virus' life cycle. Furthermore, the results of viral accumulation in the specific gene knockdown plants and protoplasts could specify that the host factor is acting in the replication or movement step of the infection cycle.

The following sections provide discussions of how the host factors identified by the techniques described above participate in BaMV replication and movement. The study of virus infection mechanisms and hosts' responses to them will provide a better understanding of the relationship between pathogens and hosts. This learning would lead to designing the better strategies for pathogen control on plants.

## THE FACTORS INVOLVED IN ASSISTING BAMV RNA REPLICATION

The entire replication processes of a positive-sense RNA virus could be divided into a few different steps. First, once BaMV enters a host cell, with bamboo as a natural host and *N. benthamiana* as an experimental host, the RNA genome is used as a template for translation to synthesize the replication enzyme, replicase (**Figure 1**). At this stage, the 3' UTR is playing a critical role in trapping a few different factors that would lead to the next step of the replication process, targeting the replication site. At least four host proteins, glutathione transferase U4 (NbGSTU4), the eEF1a, chloroplasts PGK (chlPGK), and heat shock protein 90 (Hsp90), were discovered to interact with the 3' UTR of BaMV. In particular, the eEF1a was shown to play a negative role in BaMV RNA replication (Lin et al., 2007). Since the binding site of the eEF1a in the 3' UTR of BaMV is overlapped with that of the RdRp, the eEF1a might play a role in the template switch that blocks viral replication during translation. The eEF1a has commonly been demonstrated to bind the tRNA-like structure of *Brome mosaic virus* (BMV) (Bastin and Hall, 1976) and *Turnip yellow mosaic virus* (TYMV) (Joshi et al., 1986). This interaction was claimed to function in the negative-regulation of TYMV minus-strand RNA synthesis (Matsuda et al., 2004); however, a similar interaction in *West Nile virus* was revealed to facilitate minus-strand RNA synthesis (Davis et al., 2007).

The nucleus-encoded chlPGK interacting with the 3' UTR (Lin et al., 2007) was demonstrated to play a role in ushering the viral RNA and its associated proteins, including replicase, into chloroplasts for replication (**Figure 1**) (Cheng et al., 2013).



The genomic RNA was visualized in the chloroplast by confocal microscopy after being labeled with a green fluorescent protein fusion MS2 coat protein construct (NLS-MS2-GFP) that could recognize the BaMV RNA containing the MS2 hairpins (Cheng et al., 2013). The advantage of BaMV targeting a chloroplast for replication is that it might be a way to hide from the host scavenging system, including the RNA silencing pathway. To get into the chloroplast for replication, the entire replication complex—including the viral RNA [approximately 6.4 kb plus the poly(A) tail], replicase, and other associated host factors—must be transporting into the chloroplasts through the chloroplast transporting complex. This gigantic viral RNA-protein complex can enter into the chloroplasts because Hsp90 interacting with the 3' UTR of BaMV was demonstrated to play a positive role in the very early event of BaMV replication (Huang et al., 2012b). Heat shock proteins acting as chaperones on protein complex folding, protein degradation, and protein translocation across membranes (Mayer and Bukau, 2005; Taipale et al., 2010) could help transport the viral RNP complex into the chloroplasts (Figure 1). HSPs were shown to be assisting the viral RNA recruitment and viral replication complexes (VRCs) assembly (Pogany et al., 2008; Wang et al., 2009a,b; Huang et al., 2012b). Accordingly, Hsp90 involved in the early event of BaMV replication could be implied to assist the viral RNP

complex entry into the chloroplasts and stimulate the replication complex assembly on the right location in order to initiate the minus-strand RNA synthesis.

Another 3' UTR-associated protein, NbGSTU4, was demonstrated to be upregulated post BaMV infection and involved in assisting the replication of BaMV *in vitro* and *in vivo* (Chen et al., 2013). In general, GSTs are involved in the antioxidation process; the oxidative stress triggered by a pathogen infection could be attenuated via the enzymatic reaction of GSTs. A chloroplast is one of the major reactive oxygen species (ROS) producer in which the relative concentration of ROS should be higher than that of other organelles. Once BaMV enters into the chloroplasts for replication, the viral RNAs face the obstacle of the higher levels of ROS produced either from the photosynthesis process or the virus infection. NbGSTU4 moving with BaMV RNA in the presence of Glutathione (GSH) could play a role in eliminating the effects of ROS. We have demonstrated that NbGSTU4 could interact with the 3' UTR in the physiological concentration of GSH, which is approximately 10 mM (Chen et al., 2013). These results imply that NbGSTU4 could also be one of the host proteins associated with viral RNA and was transported together into the chloroplasts.

A 5' to 3' exonuclease (XRN4), a protein component enriched in BaMV RdRp preparation, was demonstrated to

enhance the accumulation of BaMV (Lee et al., 2015). The members of the XRN\_N family are divided into two groups, the cytoplasmic (XRN1/PACMAN or XRN4) and the nuclear enzymes (XRN2/RAT1 and XRN3), and display the RNase activities for mRNA degradation (Kastenmayer and Green, 2000; Souret et al., 2004). Although XRN4 was revealed to conduct antiviral activity against *Tomato bushy stunt virus* (TBSV) and *Tobacco mosaic virus* (TMV) (Cheng et al., 2007; Jaag and Nagy, 2009; Peng et al., 2011), the presence of XRN4 could elevate the accumulation of BaMV. Because XRN4 was demonstrated to have a role in reducing the activities of siRNA- and miRNA-mediated RNA decay in *Arabidopsis* (Souret et al., 2004), downregulated the silencing pathway might result in a positive regulation of BaMV RNA accumulation.

Some host factors identified by cDNA-AFLP could influence the replication of BaMV in an indirect manner so that these proteins could not be revealed by using the strategy of detecting the direct interaction with viral products. NbRabG3f was demonstrated to be a positive regulator in BaMV replication by loss- and gain-of-function assays (Huang et al., 2016). Rabs are a group of small GTPases involved in vesicles transport, uncoating, tethering, and fusion (Seabra et al., 2002). A deletion mutant of NbRabG3f failure in membrane-anchoring lost the ability to assist the accumulation of BaMV. A mutant with the fixed GDP-bound RabG3f (T22N) was trapped at the Golgi and could not assist the accumulation of BaMV. Overall, these results suggest that NbRabG3f is involved in a vesicle budding from the Golgi and transports the cargos containing the unidentified host factors to the destination site for BaMV replication (**Figure 1**).

Based on the host factors identified so far for BaMV replication, BaMV RNA entry into the host cell would require chlPGK to usher the viral RNA into the chloroplasts. The transport of the viral RNP complex needs the chaperon Hsp90 to cross the membrane and assemble the functional replication complex. During this process of trafficking from the cytoplasm to the chloroplasts, NbXRN4 might be involved in reducing the activities of siRNA-mediated silencing. Once the viral RNP is transported into the chloroplasts, the VRC needs the anti-oxidant enzyme NbGSTU4 to neutralize the oxidative stress inside the chloroplasts for an efficient minus-strand RNA synthesis (**Figure 1**).

## THE FACTORS INVOLVED IN VIRAL RNA MOVEMENT

Through a biochemical analysis of the BaMV movement complex isolated by co-immunoprecipitation using an anti-TGBp3 antibody, the movement trafficking complex was revealed to harbor not only the MPs TGBp1, TGBp2, and TGBp3, but also the coat protein and replicase (Chou et al., 2013). The TGBps-mediated cell-to-cell trafficking was proposed to be in two possible paths: TGBps-associated virion complex traffics alongside the endoplasmic reticulum (ER) network, or the virions and the MPs would associate with the TGBp2-induced vesicles (Chou et al., 2013; Liou et al., 2015).

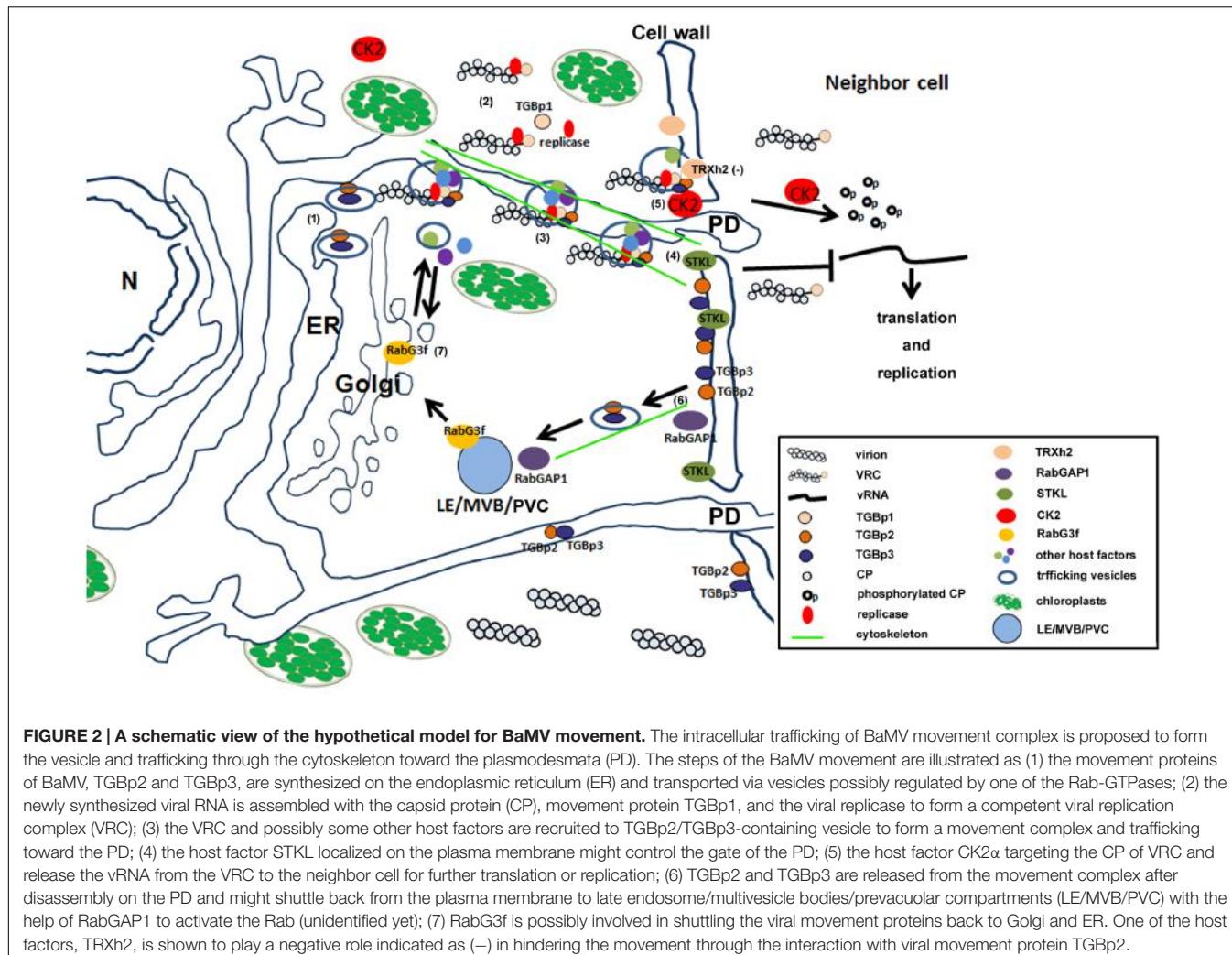
A few host factors were identified to participate in the process of BaMV movement. A RabGTPase-activating protein (GAP) designated as NbRabGAP1 was demonstrated to participate in BaMV cell-to-cell and systemic movements (Huang et al., 2013). Rabs, a family of small GTPases, are known to be involved in all aspects of intracellular vesicle budding, targeting, docking, and fusion (Johansen et al., 2009; Mizuno-Yamasaki et al., 2012; Cherfils and Zeghouf, 2013). Two Rabs regulators, guanine nucleotide exchange factors (GEFs), and GAPs play roles in recycling Rabs for vesicles trafficking, in which GEFs exchange GDP for GTP and GAPs accelerate GTP hydrolysis (Bos et al., 2007). The results of the mutational analysis of NbRabGAP1 in BaMV accumulation suggest that the fully GAP function of NbRabGAP1 is essential to support the efficient movement of BaMV. The proposed role of NbRabGAP1 in BaMV movement is that NbRabGAP1 is to trigger one of the RabGTPases (not yet identified) to release the vesicles containing the viral movement complex trafficking to the plasmodesmata (PD) (**Figure 2**), similar to those revealed in *Chinese wheat mosaic virus* (Andika et al., 2013), or to shuttle TGB proteins from the PD via the endocytic pathway back to the ER, like those found in *Potato mop top virus* (Haupt et al., 2005).

A serine/threonine kinase-like protein from *N. benthamiana* (NbSTKL), an upregulated gene that is post BaMV inoculation, was demonstrated to be critical in the movement step of the BaMV infection cycle (Cheng et al., 2013). The results from the sequence analysis and the intracellular localization indicated that NbSTKL is plasma membrane-associated through myristylation at glycine, the second amino acid from the N-terminus. The mutant that lost the kinase activity (NbSTKL/D224A) or failed to associate with the plasma membrane (NbSTKL/G2A) also failed to enhance the movement of BaMV. These results suggest that NbSTKL might target a specific factor on the membrane, regulating the gating of the PD for the passage of BaMV (**Figure 2**). Furthermore, another kinase, casein kinase 2 $\alpha$  (CK2 $\alpha$ ), which interacts with BaMV CP in PD, might assist the release of viral RNA from the RNP movement complex during the viral RNP complex passage through the PD (**Figure 2**) (Hung et al., 2014).

Taken together, the identification of NbRabGAP1 involved in the movement of BaMV supports the idea that the movement could be made through the vesicle trafficking path (Chou et al., 2013; Liou et al., 2015). To reach an efficient movement of BaMV requires at least two kinases, NbSTKL and CK2 $\alpha$ , gating the PD and releasing the viral RNA from the RNP complex (**Figure 2**). Obviously, some other factors are also required for this process such as the target of NbSTKL.

## THE FACTORS INVOLVED IN DEFENSE AGAINST VIRAL RNA REPLICATION

As mentioned previously, once a virus enters a host cell, it needs not only to seek the host factors for assistance, but also to face the challenges from the host itself. In plants, there already exist a few defense mechanisms such as the RNA silencing pathway and the elector-induced hypersensitive reaction. In addition, some novel



proteins in host cells could display anti-viral activities beyond their already known functions. A putative methyltransferase (PMTS1) once interacted with BaMV RdRp, screened by a yeast two-hybrid technique, and displayed an inhibitory effect on the RdRp activity with a dosage-dependent fashion (**Figure 1**) (Cheng et al., 2009). PMTS1 comprises an N-terminal signal peptide predicted to target mitochondria or chloroplasts and two putative AdoMet-binding motifs in the middle region. Removing the signal peptide or abolishing the AdoMet-binding activity of PMTS1 would cause the loss of its inhibitory effect. Because BaMV has been demonstrated to replicate in chloroplasts, the signal peptide of PMTS1 targeting the chloroplasts is highly recommended.

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) found in the purified RdRp complexes could bind the stem-loop C/poly(A) in the 3' UTRs of satBaMV and the pseudoknot/poly(A) in the 3' UTR of BaMV (Prasanth et al., 2011). Further analysis indicates that the cytosolic, GAPDH, could inhibit the replication of BaMV/satBaMV (**Figure 1**). The purified recombinant, GAPDH, could specifically inhibit the synthesis of minus-strand RNA of BaMV/satBaMV in an *in vitro*

replication assay. GAPDH is a multifunctional enzyme involved in quite diverse activities in cells, including glycolysis, cellular dysfunction, cell death, apoptosis, association with cytoskeleton and vesicles transport, exportation of nuclear RNA, and DNA repair (Tristan et al., 2011). In *Arabidopsis*, cytosolic GAPDH was found to be a prominent target of H<sub>2</sub>O<sub>2</sub>-dependent oxidation (Hancock et al., 2005), but could be reversible back in the presence of reductant GSH (Bedhomme et al., 2012). Although the functions of GAPDH involved in BaMV replication are not clear, the simple interaction of GAPDH with the 3' UTRs of BaMV and satBaMV could block the accessibility of RdRp for initiating the minus-strand RNA synthesis.

## THE FACTORS INVOLVED IN DEFENSE AGAINST VIRAL RNA MOVEMENT

Regarding the movement of potexviruses, both MP and CP are vital for efficient cell-to-cell movement and vascular transport (Verchot-Lubicz, 2005; Verchot-Lubicz et al., 2010). Post-translational modification, including ubiquitination,

sumoylation, glycosylation, and phosphorylation of viral proteins, were issued as parts of an important process in modulating the structures and functions of viral proteins (Barajas and Nagy, 2010; Alcaide-Loridan and Jupin, 2012; Mathur et al., 2012; Perez Jde et al., 2013; Samuilova et al., 2013; Xiong and Wang, 2013). Maintaining the protein structural integrity, including those modifications of MP and CP, is critical for virus movement.

One of the thioredoxin proteins, NbTRXh2, an upregulated gene post BaMV inoculation, was demonstrated to restrict the movement of BaMV (Chen et al., 2017). NbTRXh2 was localized at the plasma membrane through myristylation at the Glycine of the second amino acid from the N-terminus. NbTRXh2 was revealed to target the MP TGBp2 to reduce its disulfide bond (**Figure 2**). Also, the two conserved cysteins forming the disulfide bond were demonstrated to play a key role in BaMV movement (Tseng et al., 2009). Therefore, NbTRXh2 targets TGBp2, which could result in the loss of the structural integrity of TGBp2 and their failure to interact with other movement-associated proteins, including TGBps1 and 3.

## SUMMARY AND FUTURE PROSPECTIVE

Taking all the available results into account, it can be concluded that some of the host factors are unique to BaMV, while some

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of them could be applied to other viruses. Some host factors could assist virus replication and movement, but some of them are involved in resisting virus infection. This review summarized a few host factors identified with different strategies and their possible roles in BaMV infection. However, to complete an accurate understanding of BaMV infection, more host factors need to be identified. Based on our current understandings, a few processes in BaMV infection cycle are still unclear. One of the most challenges is to uncover the process of how the newly synthesized RNAs are transported out of the chloroplasts where BaMV replicates. Hopefully, a much clearer picture of the infection cycle of BaMV can be obtained in the near future by knowing how of these factors involved.

## AUTHOR CONTRIBUTIONS

Y-PH wrote the viral replication part of the manuscript. I-HC wrote the viral movement part of the manuscript. C-HT wrote the rest part and edits the manuscript.

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# Nuclear-Encoded Plastidal Carbonic Anhydrase Is Involved in Replication of *Bamboo mosaic virus* RNA in *Nicotiana benthamiana*

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On inoculation of *Nicotiana benthamiana* with *Bamboo mosaic virus* (BaMV), a gene with downregulated expression was found involved in the infection cycle of BaMV. To uncover how this downregulated gene affects the accumulation of BaMV in plants, we used loss- and gain-of-function experiments. Knockdown of this gene decreased the accumulation of BaMV coat protein to approximately 60% in both plants and protoplasts of *N. benthamiana* but had no effect on *Potato virus X* and *Cucumber mosaic virus* infection. The full-length gene was cloned and revealed as an *N. benthamiana* nuclear-encoded chloroplast carbonic anhydrase (CA) and so designated NbCA. As compared with the accumulation of BaMV RNAs in NbCA-knockdown protoplasts, both plus- and minus-strand RNAs were reduced. We further fused NbCA with Orange fluorescent protein to confirm its localization in chloroplasts on confocal microscopy. However, transiently expressed NbCA in chloroplasts did not considerably increase BaMV accumulation. The addition of exogenous CA may not have any additive effect on BaMV accumulation because of the natural abundance of CA in chloroplasts. In an *in vitro* replication assay, the addition of *Escherichia coli*-expressed NbCA enhanced exogenous template level (re-initiation and elongation) but not endogenous template level (only elongation). These results suggest that NbCA is possibly involved in re-initiation step of BaMV RNA replication. Further analysis indicated that proton concentration could influence the endogenous and exogenous template activities. Hence, our results implied that NbCA could be playing a role in harnessing proton concentration and favoring the replicase with the re-initiation template.

**Keywords:** carbonic anhydrase, *Bamboo mosaic virus*, *Nicotiana benthamiana*, RNA replication, *in vitro* replication, initiation/elongation switch

## INTRODUCTION

*Bamboo mosaic virus* (BaMV), belonging to the *Potexvirus* genus of family *Alphaflexiviridae* (Lin et al., 1994), has one single-stranded positive-sense RNA genome of approximately 6.4 kb long [excluding the poly(A) tail]. The genome comprises a 5' cap structure, 3' poly(A) tail, and five open reading frames (ORFs 1–5) (Lin et al., 1994). ORF1, encoding a 155-kDa polypeptide, harbors three

functional domains (Meng and Lee, 2017): the capping enzyme domain, which exerts AdoMet-dependent guanylyltransferase activity (Li et al., 2001a; Huang et al., 2004; Hu et al., 2011); the helicase-like domain, which contains NTPase and RNA 5'-triphosphatase activities (Li et al., 2001b); and the RNA-dependent RNA polymerase (RdRp) core domain (Li et al., 1998; Cheng et al., 2001). ORFs 2–4, encoding 28-, 13-, and 6-kDa polypeptides, respectively, overlap and are called triple-gene-block (TGB), designated TGBp1, -2, and -3, respectively. The movement of BaMV requires these three TGB proteins (Lin et al., 2004, 2006; Chou et al., 2013). ORF5, encoding a 25-kDa polypeptide viral capsid protein (CP) is required for cell-to-cell movement, symptom development, and virion assembly (Lan et al., 2010; Hung et al., 2014a,b). The 3' untranslated region (UTR) plays roles in minus-strand RNA initiation, polyadenylation, and long-distance movement (Chen et al., 2017).

Although bamboo is the natural host for BaMV, *Nicotiana benthamiana* is the major assay host for studying the infection cycle of BaMV at the molecular level. A putative methyltransferase was identified to play a role in restricting the accumulation of BaMV in a dose-dependent manner in protoplasts (Cheng et al., 2009). Glyceraldehyde 3-phosphate dehydrogenase was found to play an inhibiting role in regulating minus-strand RNA synthesis by binding to the 3' UTR of BaMV RNA (Prasanth et al., 2011). The chloroplast phosphoglycerol kinase (PGK) interacts with the 3' UTR, including part of the poly(A) tail, and ushers the viral RNA into the chloroplast for BaMV replication (Lin et al., 2007; Cheng et al., 2013a). A heat shock protein 90 homolog binds to the viral replicase, and the 3' UTR enhances the early stage of BaMV replication (Huang et al., 2012). A glutathione transferase, NbGSTU4, interacts with the 3' UTR of BaMV RNA and enhances the minus-strand RNA synthesis (Chen et al., 2013). Another viral replicase-associated host protein, XRN4, with RNase activity, assists the accumulation of BaMV (Lee et al., 2015). NbRabG3f, an Rab-GTPase protein, is involved in positive regulation of BaMV replication (Huang et al., 2016). The host factor Ser/Thr kinase-like protein (NbSTKL), localized mainly on the cell membrane, can facilitate BaMV intercellular movement (Cheng et al., 2013b). An RabGTPase-activating protein (NbRabGAP1) is involved in BaMV cell-to-cell and systemic movement (Huang et al., 2013).

Carbonic anhydrase (CA) is a zinc metalloenzyme that can catalyze the interconversion of carbon dioxide ( $\text{CO}_2$ ) and bicarbonate ( $\text{HCO}_3^-$ ). The reaction of  $\text{CO}_2 + \text{H}_2\text{O} \leftrightarrow \text{HCO}_3^- + \text{H}^+$  reaches equilibrium spontaneously but slowly and can be accelerated by the catalyzation of CA (Dimario et al., 2017). CA also plays vital roles in many biochemical processes that involve pH homeostasis and ion transport (Tashian, 1989) and carboxylation or decarboxylation reactions such as photosynthesis and respiration, respectively (Moroney et al., 2001). From the structures and amino acid sequences, CAs can be divided into five distinct classes:  $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$ , and  $\epsilon$ , which share little sequence similarity and are assumed to have evolved independently (Hewett-Emmett and Tashian, 1996; Tripp et al., 2001; So

et al., 2004; Sawaya et al., 2006; Floryszak-Wieczorek and Arasimowicz-Jelonek, 2017). The CAs of algae and plants are all belong to  $\alpha$ ,  $\beta$ , and  $\gamma$  classes, with the  $\beta$  class most prevalent (Moroney et al., 2001). Furthermore, in C3 plants such as *N. benthamiana*, CA is found in the stroma of mesophyll chloroplasts and has been found with some characteristics such as the ability to bind salicylic acid (SA), antioxidant activities in response to pathogens (Slaymaker et al., 2002; Restrepo et al., 2005), the provision of  $\text{HCO}_3^-$  for lipid biosynthesis (Hoang and Chapman, 2002) and the regulation of  $\text{CO}_2$ -mediated stomatal closure (Hu et al., 2010).

The relation between CA and plant pathogens has been studied lately. CA is identified as a SA-binding protein 3 (SABP3) and exhibits CA enzymatic, SA-binding, and antioxidant activities in *N. tabacum* plants. Furthermore, reducing the expression of CA in plants suppressed the hypersensitive reaction (HR) in disease resistance (Slaymaker et al., 2002). In CA-silenced *N. benthamiana* plants, the growth of *Phytophthora infestans* was considerably increased, probably also due to suppression of the HR (Restrepo et al., 2005).

Although this earlier research mostly documented that the host CA is necessary for plant defense, in this study, we found that CA could, by contrast, help BaMV accumulation. Therefore, we investigated how CA could play a role in assisting BaMV accumulation in plants. Furthermore, we studied whether CA is involved in the initiation step of BaMV replication in *N. benthamiana*.

## MATERIALS AND METHODS

### Plants and Viruses

*Nicotiana benthamiana* plants were grown in a growth room at 28°C with 16 h light and 8 h dark. Three viruses were used for inoculation: BaMV strain S (Lin and Hsu, 1994), *Potato virus X* (PVX) strain Taiwan, and *Cucumber mosaic virus* (CMV) strain NT9 (Hsu et al., 1995).

### Virus-Induced Gene Silencing (VIGS) and Mechanical Inoculation of Viruses

The cDNA fragment of ACAC10-1 (fragment of *NbCA* gene) was cloned into the pGEM-T Easy vector (Promega, Madison, WI, United States) in a previous study (Cheng et al., 2010). To use ACAC10-1 in *Tobacco rattle virus* (TRV)-based VIGS (Liu et al., 2002) in *N. benthamiana* plants, ACAC10-1 in the pGEM-T Easy vector was digested with EcoRI and subcloned into the pTRV2 vector to generate pTRV2-NbCA and transformed into *Agrobacterium* C58C1 strain. Furthermore, *Agrobacterium* carrying a pTRV2-containing luciferase (*Luc*) gene or phytoene desaturase (PDS) gene were used as a negative or positive control, respectively. *Agrobacterium* containing pTRV1, pTRV2-NbCA, pTRV2-Luc, or pTRV2-PDS was cultured at 30°C to OD<sub>600</sub> 0.8~1.0; the cells were collected by centrifugation at 5000 rpm, then suspended in the induction medium (10 mM MgCl<sub>2</sub>, 10 mM MES pH5.6, and 150 μM acetosyringone) at 30°C for 1 h. After induction, equal volumes of both cultures (pTRV1 and pTRV2-NbCA, pTRV2-Luc or pTRV2-PDS) were mixed

before agroinfiltration onto the second, third, and fourth leaf of true leaves of a 1-month old *N. benthamiana*. When the PDS-knockdown plants had a photo-bleach phenotype, 500 ng of BaMV virion was mechanically inoculated onto the fourth leaf above the infiltrated leaves.

The knockdown efficiency of *NbCA* was calculated by measuring the expression ratio normalized to the expression of *actin* between the *NbCA*-knockdown and control plants. Two sets of primers were used for the RT-PCR to amplify *NbCA* and *actin* gene expression: *NbCA/F* (5'-A GTGCATGTGGAGGTATCAAAGGT-3')/*NbCA/R* (5'-GTCG ACTACGGAAAGAGAAGG-3') and *actin/3'* (5'- GTGG TTTCATGAATGCCAGCA-3')/*actin/5'* (5'-GATGAAGATAC TCACAGAAAGA-3').

## Protoplast Preparation and Viral RNA Inoculation

The preparation of protoplasts from *N. benthamiana* and viral RNA inoculation was described previously (Tsai et al., 1999). Approximately 2 g of agroinfiltrated leaf was collected from the knockdown *N. benthamiana* and digested with pectinase and cellulase at 25°C overnight. The mesophyll protoplasts were isolated from the interface zone between the Mannitol-MES buffer and the sucrose. After a few washes, protoplasts were stained with fluorescein diacetate to examine the quality of cells under a fluorescent microscope. Approximately  $2.5 \times 10^5$  protoplasts were inoculated with 1.5 µg BaMV, PVX, or CMV viral RNA with 40% polyethyleneglycol-6000. Total protein or RNA was extracted from protoplasts and detected by western blot analysis or real-time qRT-PCR, respectively.

## Western Blot Analysis

The total protein of inoculated leaves or protoplasts was extracted with plant extraction buffer (50 mM Tris-HCl pH 6.8, 10% glycerol, and 2% SDS), boiled with the SDS sample buffer (10% glycerol, 12.5 µg/ml bromophenol blue, 10 mg/ml SDS, 125 mM Tris-HCl pH 6.8, 2.5% β-mercaptoethanol) for 5 min, separated on a 12% polyacrylamide gel containing 0.1% SDS, transferred onto a nitrocellulose membrane (PROTRAN BA 85 Schleicher and Schnell), and probed with primary antibody [anti-Orange fluorescent protein (OFP), -BaMV, -PVX, or -CMV] and with the secondary antibody [affinity purified anti-rabbit IgG conjugated IRDye 800 (ROCKLAND)]. Finally, membranes with fluorescent bands were scanned by using LI-COR Odyssey (LI-COR Biosciences). In addition, rbcL (RuBisCo large subunit) stained with Coomassie blue was used as a loading control.

## Total RNA Extraction

Total RNA was extracted from leaves with STE buffer (400 mM Tris-HCl pH 8.0, 400 mM NaCl, and 40 mM EDTA), 1% SDS, and 3.3 mg/ml bentonite and an equal volume of phenol/chloroform. After ethanol precipitation, the RNA was further precipitated with 3 M NH<sub>4</sub>OAc, washed, dried, and dissolved in 30 µl de-ionized H<sub>2</sub>O. For RNA extracted from protoplasts, cells were mixed with 200 µl protoplast RNA extraction buffer (100 mM

Tris-HCl pH 8.0, 10 mM EDTA, 100 mM NaCl, 1% SDS, and 600 µg bentonite). After phenol/chloroform extraction and ethanol precipitation, RNA was further precipitated with 3 M NH<sub>4</sub>OAc and dissolved in 13 µl de-ionized H<sub>2</sub>O.

## qRT-PCR

qRT-PCR was used to detect both BaMV plus- and minus-strand genomic RNA. The cDNA synthesis reaction involved use of ImProm-II Reverse Transcriptase (Promega, Carlsbad, CA, United States) as instructed with the primers for Oligo dT(25T) and BaMV+51 (5'-ACTGCCATTGCCCCCTACA-3') for the plus- and minus-strand, respectively. For quantifying the accumulation of BaMV genomic RNA or minus-strand RNA, primers for BaMV+51 and BaMV-282 (5'-TGTGCTGAACGGGTTATGAG-3') or BaMV+1766 (5'-CACATCCGGCACTTACCA-3') and BaMV-2002 (5'-AT GTATCACGGAAATAAGAGTT-3') were used, respectively, in the reaction containing a 1000X dilution of SYBR green I (Cambrex Bio Science Rockland, ME, United States). qPCR was performed in 0.2-ml PCR tubes with 0.6 mM primer, 0.2 mM each deoxyribonucleoside triphosphate, 10 mM Tris-HCl pH 8.8, 1.5 mM MgCl<sub>2</sub>, 50 mM KCl, 0.1% Triton X-100, 2 µl cDNA, 3 units of Taq DNA polymerase (Promega) and RNase-free water to a final volume of 20 µl. Cycling conditions began with an initial hold at 95°C for 5 min, followed by about 30 cycles of 94°C for 30 s, 56°C for 30 s and 72°C for 30 s. Reactions were carried out in a RotorGene 3000 (Corbett Research, Sydney, Australia) with data acquisition at 72°C on the channel, excitation at 470 nm and detection at 585 nm, by using a high-pass filter for both plus- and minus-strand. The reaction without template or reverse transcriptase was a negative control, and *actin* was detected for normalization. All samples were run at least three times.

## NbCA Cloning and Visualizing Its Localization

The full-length CA cDNA of *N. benthamiana* was cloned into the pEpyon binary vector that carries the *mOrange2* reporter gene (Shaner et al., 2008) to express the fusion protein NbCA-OFP. The ORF of the *NbCA* was amplified with *NbCA/F*, 5'-GGATCCATGTCAACTGCTTCCA-3', and *NbCA/R*, 5'-GGTACCTACGGAAAGAGAAG-3' (*Bam*HI and *Kpn*I underlined, respectively). The PCR product was first cloned into the pGEM-T easy vector (Promega, Madison, WI, United States), then sub-cloned into pEpyon with *Bam*HI and *Kpn*I after sequence verification.

*Agrobacterium* containing the binary vector with NbCA-OFP or vector alone was cultured and infiltrated into *N. benthamiana* plants. The fluorescent signals were detected at 3 days post-infiltration by confocal laser scanning microscope (FV1000, Olympus). To observe whether NbCA altered its localization after BaMV infection, pKBG, a plasmid containing an infectious cDNA of BaMV with a GFP reporter (Prasanth et al., 2011) was co-infiltrated with NbCA-OFP. The fluorescent signals were detected at 4 days post-infiltration by confocal laser scanning microscope.

## Transient Expression of NbCA-OFP Fusion Protein

NbCA-OFP was transiently expressed via agroinfiltration on a leaf for 1 day and the BaMV virion was inoculated on the same leaf for another 3 days. The expression of this fusion protein and accumulation of BaMV coat protein were detected by western blot analysis.

## NbCA Expression and Purification from *Escherichia coli*

The coding sequence of *NbCA* without the predicted transit peptide (Slaymaker et al., 2002) was amplified with the primer set CAgene/F, 5'-GGATCCGAATTGCAATCATCAGATGG-3', and CAgene/R, 5'-GCTCGAGTACGGAAAGAGAAGGAGAAA-3' (*Bam*H I and *Xba*I site underlined, respectively). The PCR product was cloned into the pGEM-T easy vector and the sequence was verified. Finally, *NbCA* was subcloned from the T-vector into the pET29a(+) expression vector (Invitrogen) and transformed into *E. coli* BL21(DE3). The resulting clone was designated pET29a(+)-NbCA.

*Escherichia coli* containing pET29a(+-NbCA was cultured to OD<sub>600</sub> = 0.7 ~ 1.2 (100 ml in total volume), the expression was induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at 16°C for 1 day, then samples were centrifuged at 7000 rpm at 4°C for 7 min. The cell pellet was resuspended with 8 ml buffer A (50 mM NaH<sub>2</sub>PO<sub>4</sub> pH 8.0, 300 mM NaCl) containing protease inhibitor cocktail (Roche, Germany) and subjected to the French Press to break cells: the sample was loaded into the French Press and squeezed out four times, then centrifuged at 12000 rpm at 4°C for 10 min. The supernatant was incubated with 1 ml complete His-tag Purification Resin (Roche, Germany) overnight, washed with 10 ml buffer A containing 50 mM imidazole, and eluted with buffer A containing 250 mM imidazole. Finally, the eluted protein was dialyzed with 150 ml buffer A four times to remove imidazole and stored at -80°C with the addition of final 10% glycerol. The vector-only construct was manipulated under the same condition as the negative control.

## Replicase Complex Preparation and *in Vitro* Replication Assay

*Bamboo mosaic virus*-infected leaves were collected at 5 dpi and homogenized with polytron in replicase complex extraction buffer (50 mM Tris-HCl pH 7.6, 15 mM MgCl<sub>2</sub>, 120 mM KCl, 0.1% β-mercaptoethanol, 20% glycerol, 1 μM pepstatin A, 0.1 mM phenylmethylsulfonyl fluoride) with a 2 ml/g of buffer/leaf ratio. The leaf slur was filtrated through Miracloth (Calbiochem) and centrifuged at 500 × g for 10 min to remove the cell debris. The pellet was resuspended in suspension buffer (50 mM Tris-HCl pH 8.2, 10 mM MgCl<sub>2</sub>, 1 mM dithiothreitol, 1 μM pepstatin A, 1 μM leupeptin) after centrifugation at 30,000 × g for 35 min. Approximately 2 ml of the extract was loaded on 28 ml of 20 ~ 60% continuous gradient of sucrose with the gradient buffer (50 mM Tris-HCl pH 8.0, 10 mM NaCl, 1 mM EDTA, 5% glycerol, 1 μM pepstatin A, 0.1 mM PMSF, 1 mM dithiothreitol) and centrifuged at 72,100 × g for 7.2 h. The 5th

and 6th fractions of the 10 fractions (from top to bottom) with the highest RdRp activity were pooled and stirred with 1.5% NP-40 for 1 h to solubilize the membrane-associated RdRp.

For the *in vitro* replication assay with the endogenous RNA templates, 15 μl of the replicase complex preparation (pH 8.0) was added to a total 50 μl reaction containing 2 mM (A, C, and G) TP, 2 μM UTP, 3 mM MgCl<sub>2</sub>, 10 mM dithiothreitol, 50 mM Tris-HCl pH 8.2 (6.8, 7.4, 7.8, 8.8, or 9.0 was used in testing the proton concentration for the *in vitro* replication assays), 12 unit RNase OUT (Invitrogen, Carlsbad, CA, United States), 8 mg bentonite, 0.066 μM [α-<sup>32</sup>P]UTP (3000 Ci mmol/1, Dupont-NEN) and 5.2 μg recombinant NbCA at 30°C for 1 h (the reaction made up of final pH is 8.1 when reaction buffer is 8.2). Therefore, the Tris-HCl buffer at different pH was used in the reaction to reach the final target pH as 7.1, 7.5, 7.8, 8.6, or 8.8, respectively. The reaction was stopped by adding 150 μl 5 mM EDTA, extracted with phenol/chloroform, and precipitated with ethanol. The radioactive RNA products were resolved on a 1% agarose gel and quantified by using the PhosphoImaging analyzer BAS-2500 (FUJIFILM).

For the exogenous RNA templates, 15 μl of the replicase complex preparation was first treated with 10 units of micrococcal ribonuclease containing 2.5 mM Ca(OAc)<sub>2</sub> to remove endogenous RNA at 30°C for 30 min in a total volume of 11.5 μl reaction. Then the reaction was terminated by adding 16 mM EGTA and set on ice for 1 ~ 2 min. An aliquot of 15 μl mixture was subjected to a total 50 μl reaction as in the endogenous RNA template reaction. The radioactive RNA products were resolved on a 5% polyacrylamide gel and quantified by using the PhosphoImaging analyzer BAS-2500 (FUJIFILM).

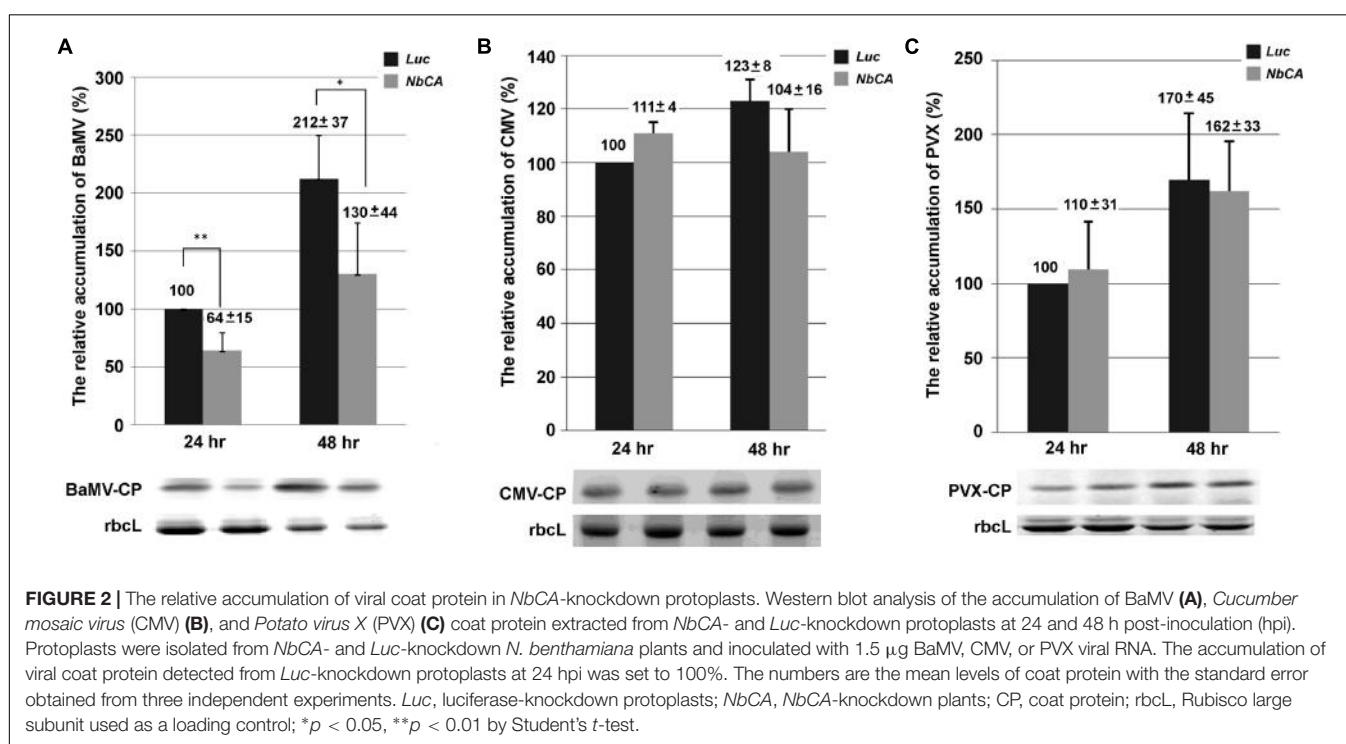
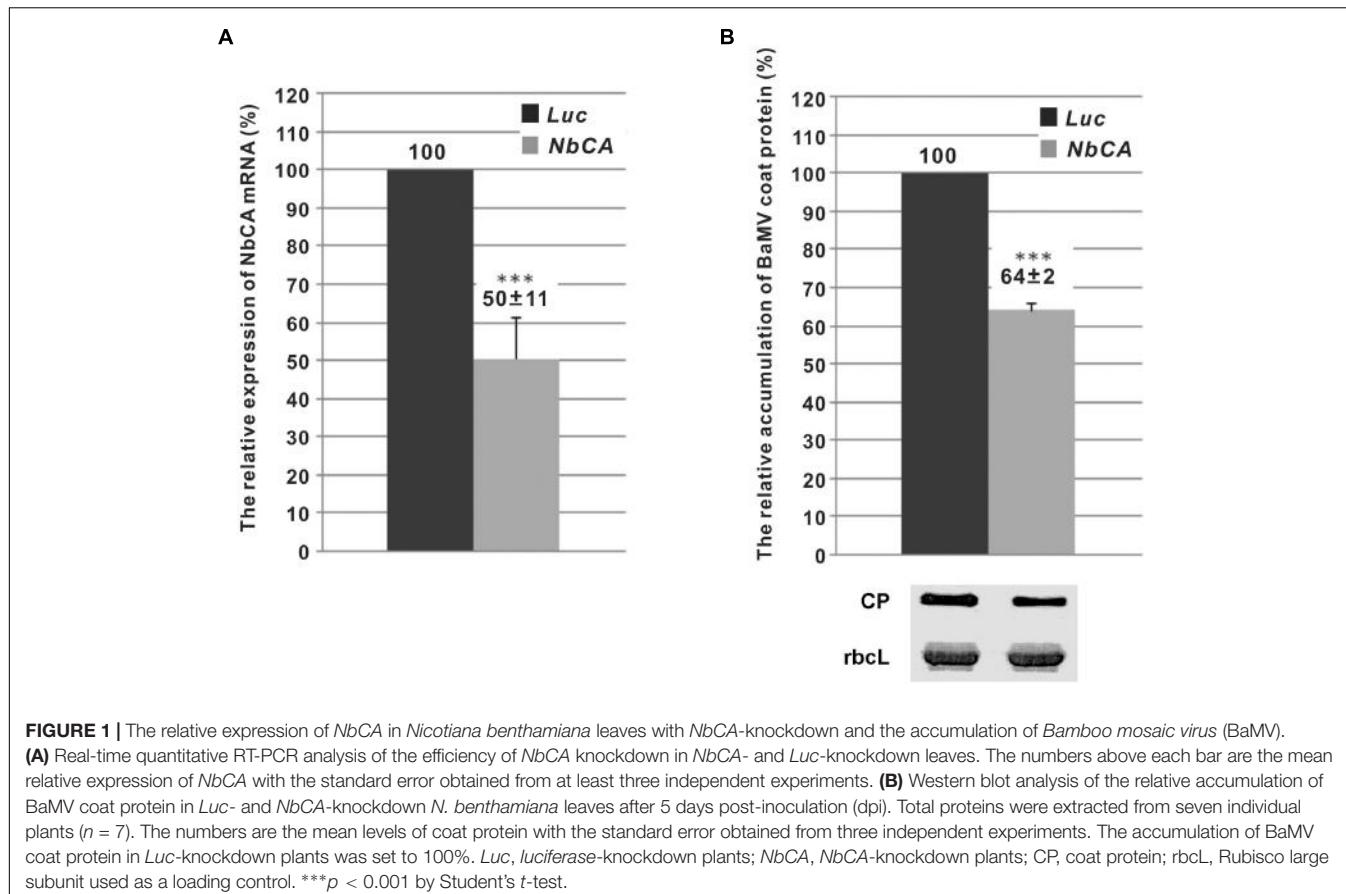
## RNA Preparation

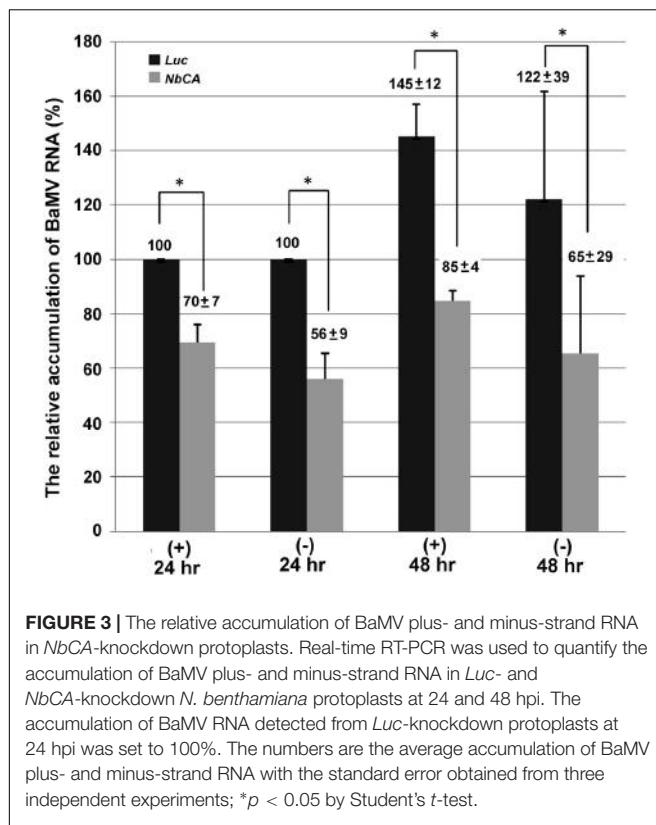
Ba-77 RNA (the 3'-end 77 nt of BaMV minus-strand RNA) and r138/40A RNA (the 3' UTR of BaMV RNA) was prepared in an *in vitro* transcription with plasmids constructed previously (Cheng et al., 2001). The reaction was carried out in 100 μl containing 10 μg linearized plasmid (*Eco*RI and *Bam*H I for Ba-77 and r138/40A, respectively, in pUC18), 40 mM Tris-HCl pH 8.0, 2 mM spermidine, 8 mM MgCl<sub>2</sub>, 10 mM dithiothreitol, 0.4 mM NTP and 200 U T7 RNA polymerase at 37°C for 2 h. The RNA was then gel purified, quantified, and stored at -80°C.

## RESULTS

### The Accumulation of BaMV in NbCA-Knockdown Plants Is Reduced

The sequence of a gene, ACAC10-1, found downregulated in *N. benthamiana* plants by cDNA-amplified fragment polymorphism (AFLP) after BaMV inoculation (Cheng et al., 2010), showed 100% match with an EST clone 30F62 containing a nuclear-encoded chloroplast CA gene. The gene was designated *NbCA*. To gain a better understanding of the relation between *NbCA* and BaMV infection, we inoculated BaMV virion into leaves of *N. benthamiana* with *Tobacco rattle virus* (TRV)-based *NbCA* knockdown.





**FIGURE 3 |** The relative accumulation of BaMV plus- and minus-strand RNA in *NbCA*-knockdown protoplasts. Real-time RT-PCR was used to quantify the accumulation of BaMV plus- and minus-strand RNA in *Luc*- and *NbCA*-knockdown *N. benthamiana* protoplasts at 24 and 48 hpi. The accumulation of BaMV RNA detected from *Luc*-knockdown protoplasts at 24 hpi was set to 100%. The numbers are the average accumulation of BaMV plus- and minus-strand RNA with the standard error obtained from three independent experiments; \* $p < 0.05$  by Student's *t*-test.

The reduced *NbCA* expression in *N. benthamiana* did not cause any morphological change as compared with control plants (infiltrated with a TRV vector-carrying *luciferase* gene) (Supplementary Figure S1). The mRNA level of *NbCA* in *NbCA*-knockdown plants was approximately 50% that in the control plants (Figure 1A). At 5 days post-inoculation (dpi), the accumulation of BaMV in *NbCA*-knockdown plants was reduced to 64% that in *Luc*-knockdown control plants (Figure 1B). Hence, *NbCA* could be a positive regulator for BaMV infection in *N. benthamiana*.

### The Requirement of NbCA for Viral Replication Is Specific to BaMV

To determine whether the role of *NbCA* for BaMV accumulation is involved in virus replication or movement, cell wall-excluded protoplasts were prepared for viral RNA inoculation to eliminate the involvement of viral movement. The accumulation of BaMV coat protein in *NbCA*-knockdown protoplasts was reduced to 64 and 61% that of control protoplasts at 24 and 48 h post-inoculation (hpi), respectively (Figure 2A). To determine whether the involvement of *NbCA* is specific to the BaMV infection cycle, CMV and PVX were inoculated into *NbCA*-knockdown protoplasts. The accumulation of the coat protein of these two viruses in knockdown protoplasts did not differ from that in control protoplasts at 24 and 48 hpi (Figures 2B,C).

Furthermore, to elucidate whether this deficiency results from a defect in synthesizing the plus- or minus-strand viral RNA,

qRT-PCR was used to quantify the accumulation of BaMV RNAs in knockdown protoplasts. At 24 hpi, the accumulation of the plus- and minus-strand of BaMV RNA in *NbCA*-knockdown protoplasts was reduced by approximately 30 and 44% as compared with control protoplasts. The similar reduced ratio in both plus- and minus-strand of BaMV RNA was observed at 48 hpi (42 and 47%, respectively). Therefore, the accumulation of plus- and minus-strand BaMV RNAs were similarly affected by the reduction in *NbCA* levels (Figure 3). These results suggest that *NbCA* is most likely involved in BaMV replication.

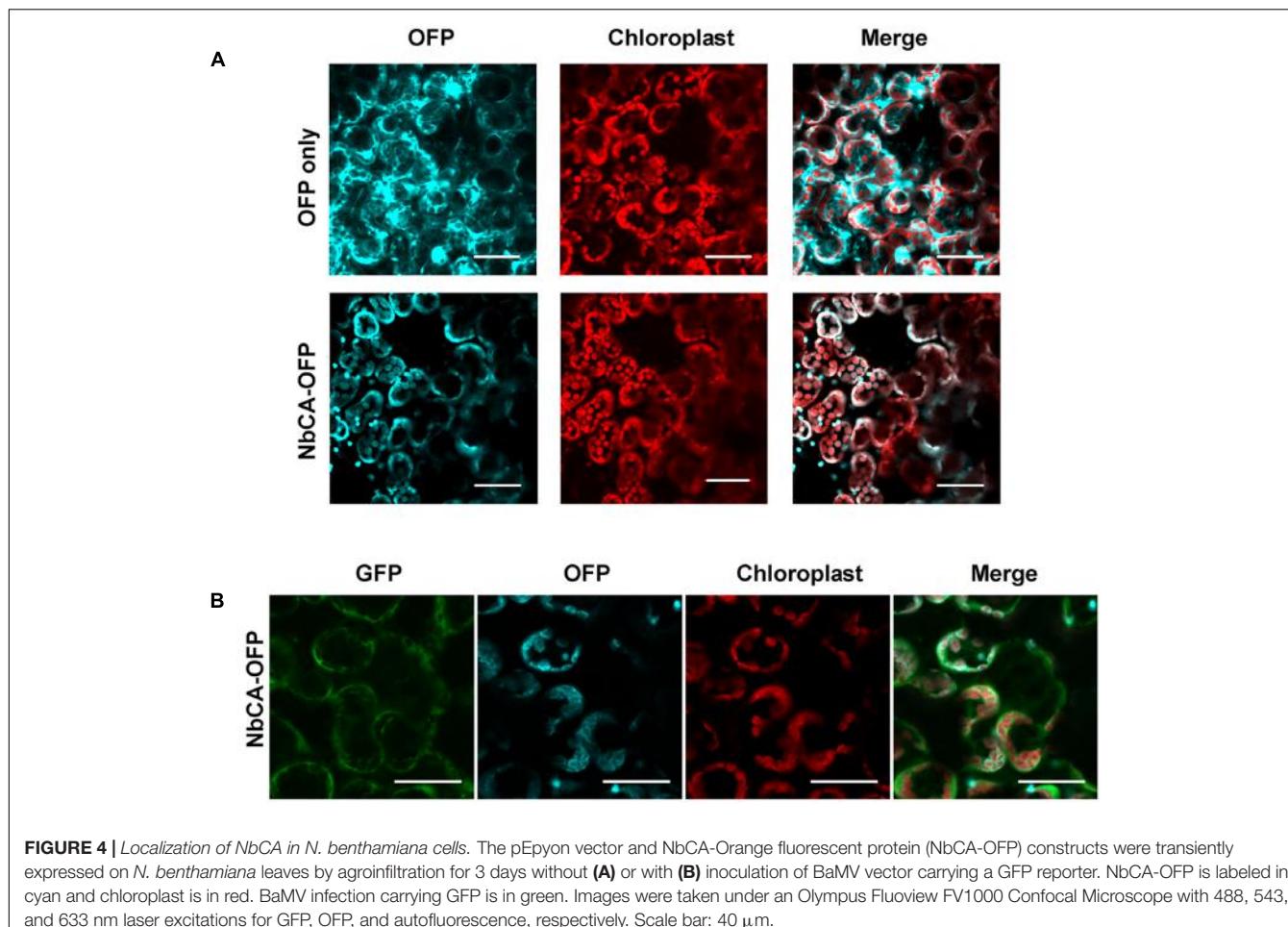
### NbCA Is Localized in *N. benthamiana* Chloroplasts

To clone the *NbCA* full-length gene, a primer was designed for the 3' rapid amplification of cDNA ends (RACE) experiment to obtain the downstream sequence of ACAC10-1. The cDNA fragment derived from 3' RACE contains the stop codon of *NbCA*. The upstream sequence of ACAC10-1 including the start codon of *NbCA* was retrieved from the transcriptome of the *N. benthamiana* draft genome (Hewett-Emmett and Tashian, 1996; Bombarély et al., 2012). Two specific primers were used to amplify the full-length *NbCA* coding region and cloned into the pEpyon binary vector (Chen et al., 2011), which carries the *mOrange2* reporter gene (OFP), to produce the *NbCA*-OFP fusion protein. Furthermore, the amino acid sequence of *NbCA* (accession no.: MF346699) was aligned with those from *N. tabacum* (NtCA; accession no.: P27141), and *Arabidopsis* (AtCA; accession no.: NP\_186799) (Supplementary Figure S2). The sequence of *NbCA* shared 97 and 68% identity with those of NtCA and AtCA, respectively.

To visualize the localization of *NbCA* in plant cells, *NbCA*-OFP was transiently expressed in *N. benthamiana* leaves by agroinfiltration to detect the fluorescent signal emitted from the OFP merged with the autofluorescence signal emitted from chloroplasts (Figure 4). *NbCA* was mainly localized in chloroplasts. Moreover, to observe whether the localization of *NbCA* was altered after BaMV inoculation, we co-infiltrated the infectious BaMV viral vector pKBG carrying green fluorescent protein (GFP) driven by subgenomic RNA promoter (Prasanth et al., 2011) with *NbCA*-OFP and found no re-localization of *NbCA* after BaMV inoculation (Figure 4B).

### NbCA Enhances BaMV Replication in Vitro

Since we found that the accumulation of BaMV coat protein and viral RNA was reduced in *NbCA*-knockdown plants (Figure 1) and protoplasts (Figures 2, 3), *NbCA* may assist viral RNA replication. To validate this hypothesis, we transiently expressed *NbCA*-OFP in *N. benthamiana* followed by BaMV inoculation. However, accumulation of BaMV coat protein was not enhanced at 3 dpi. The pool of *NbCA* in cells may be enough for BaMV replication and the addition of exogenous *NbCA* by transient expression might not provide additional help for BaMV accumulation. Hence, we used *in vitro* replication (Cheng et al., 2001; Lin et al., 2005b) to exclude the effect of sufficient amount of CA in chloroplasts. We cloned and expressed the full-length



**FIGURE 4 |** Localization of NbCA in *N. benthamiana* cells. The pEpyon vector and NbCA-Orange fluorescent protein (NbCA-OFP) constructs were transiently expressed on *N. benthamiana* leaves by agroinfiltration for 3 days without (**A**) or with (**B**) inoculation of BaMV vector carrying a GFP reporter. NbCA-OFP is labeled in cyan and chloroplast is in red. BaMV infection carrying GFP is in green. Images were taken under an Olympus Fluoview FV1000 Confocal Microscope with 488, 543, and 633 nm laser excitations for GFP, OFP, and autofluorescence, respectively. Scale bar: 40  $\mu$ m.

CA in *E. coli* to acquire the purified-NbCA for *in vitro* replication experiments. The *E. coli* BL21 (DE3)-expressed recombinant NbCA-His was purified through a Nickel-chelating resin column (**Figure 5A**).

First, we tested whether NbCA affects endogenous RNA template activity, which represents the elongation step of BaMV replication. Viral RNA synthesis did not differ with or without the addition of the *E. coli*-expressed NbCA in the replication assay (**Figure 5B**). Second, we tested whether NbCA is involved in the initiation of BaMV replication. In the *in vitro* replication, we tested the two RNA templates, r138/40A (the 3' UTR of BaMV, the promoter for minus-strand RNA synthesis) (Cheng et al., 2001) and Ba-77 (the 3'-end 77 nt of the minus-strand genome, the promoter for plus-strand RNA synthesis) (Lin et al., 2005a). The addition of NbCA in the *in vitro* replication assay with the exogenous templates r138/40A and Ba-77 increased RNA synthesis to 150 and 120%, respectively, that with vector alone (**Figures 5C,D**).

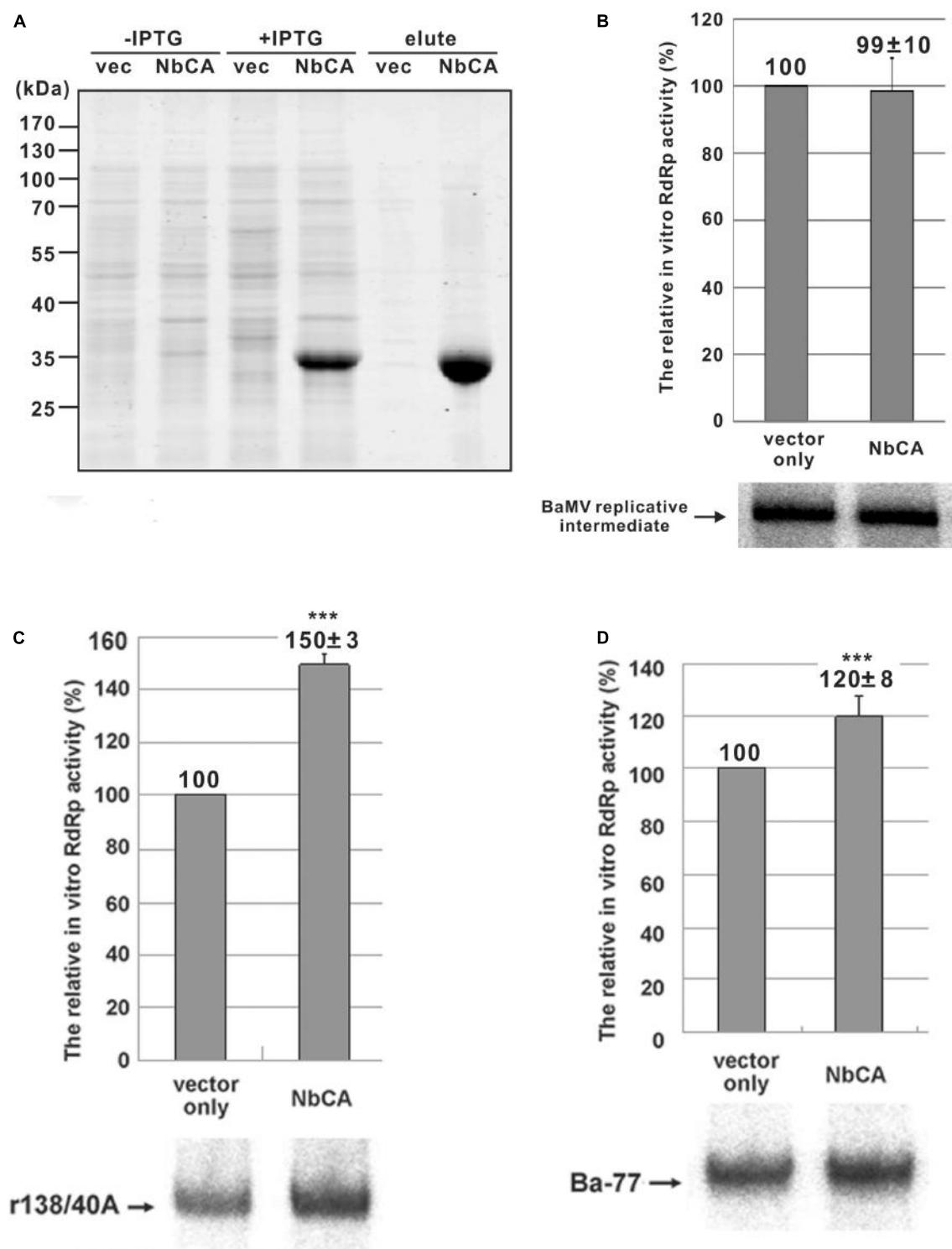
## BaMV Replication Could Be Regulated by the Proton Concentration

Carbonic anhydrase activity condenses carbon dioxide with water to produce a free proton in the reaction. We wondered whether

the proton concentration affects the viral RNA replication. In the *in vitro* replication assay, CA affected the exogenous but not the endogenous template activities. If the CA activity provides the free proton to change the micro-environment (reducing pH) such as the membrane-housed viral replication site, the condition for the re-initiation of the plus- or minus-strand RNA temples by BaMV replicase complex could be regulated. To test this hypothesis, we used various pH conditions for *in vitro* replication assays with endogenous and exogenous templates. The endogenous template (the viral RNAs already on the replicase complex and presumably at the elongation step) favored a higher pH condition (**Figure 6**). By contrast, the exogenous template (endogenous templates was removed by micrococcal nuclease and presumably at the re-initiation step) favored a lower pH condition. These results are implying that CA might be trapped into the viral replication site to produce free protons to create a more acidic microenvironment favoring the re-initiation of viral RNA replication.

## DISCUSSION

In C4 plants, CA is mainly found in the cytoplasm and involved in converting CO<sub>2</sub> into bicarbonate for carbon fixation

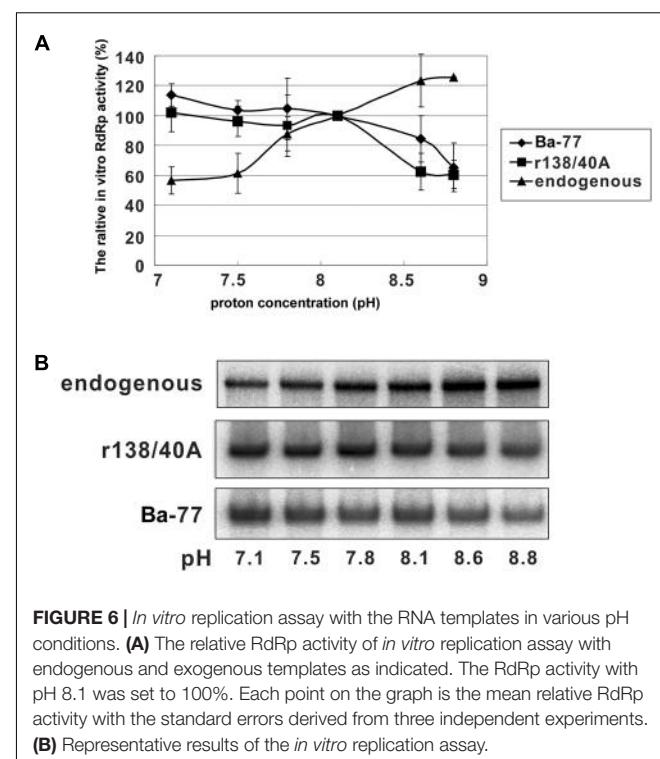


**FIGURE 5 |** The expression of NbCA in *Escherichia coli* and *in vitro* replication assays with endogenous and exogenous templates. **(A)** Total proteins were extracted from *E. coli* that expressed vector only (vec) or NbCA with or without the induction of IPTG as indicated, separated on a 12% polyacrylamide gel/SDS, and stained with Coomassie blue. The elutes indicated as elute were the total proteins eluted from His-tag purification resin. *In vitro* replication assay involved use of the purified replicase complex from infected plants with the addition of *E. coli*-expressed proteins **(A)** to test the RdRp activity of the endogenous templates **(B)** and exogenous templates r138/40A **(C)** and Ba-77 **(D)**. RdRp activity with the addition of *E. coli*-expressed eluent of vector only was set to 100%. Representative results are shown. The numbers shown above each bar are the mean relative RdRp activity with the standard errors derived from at least three independent experiments. \*\*\* $p < 0.001$  by Student's *t*-test.

(Hatch and Burnell, 1990). By contrast,  $\beta$ -CA activity is found mostly in the stroma of mesophyll chloroplasts in C3 plants (Poincelot, 1972), where it can represent up to 2% of total leaf protein (Okabe et al., 1984; Peltier et al., 2006). However, using antisense RNA to reduce this abundant chloroplast CA in C3 plants had only a marginal effect on  $\text{CO}_2$  assimilation as well as phenotypic changes (Price et al., 1994), which we observed (Supplementary Figure S1). Although the full-length CA of *N. benthamiana*, a C3 plant, has not yet been characterized, we obtained the coding region of NbCA, which showed 95% identity with *NtCA* (Supplementary Figure S2), and generated the NbCA-OFP to show chloroplast localization as predicted (**Figure 4**) (Fett and Coleman, 1994). Even though most research has revealed that CA suppression might reduce the HR response and thereby increase the susceptibility of pathogens (Slaymaker et al., 2002; Restrepo et al., 2005), CA positively regulated BaMV replication.

Carbonic anhydrase of alfalfa or tobacco was able to complement  $\Delta NCE103$ , the *Saccharomyces cerevisiae* CA-like gene deletion strain sensitive to an oxidized environment such as in the presence of  $\text{H}_2\text{O}_2$ ; hence, these two CAs were found to exhibit antioxidant activities (Gotz et al., 1999). Furthermore, *NtCA* exhibited enzymatic and antioxidant activities and also a salicylic acid-binding ability and was further called salicylic acid-binding protein 3 (SABP3) (Gotz et al., 1999; Slaymaker et al., 2002). One of the SABPs (designated SABP1) was identified as a cytosolic (peroxisomal) tobacco catalase, which exhibits  $\text{H}_2\text{O}_2$ -degrading activity (Chen et al., 1993a,b; Conrath et al., 1995). Accordingly, SABP3/*NtCA* or NbCA may also have antioxidant ability to degrade  $\text{H}_2\text{O}_2$  and then dampen the load of host defense. Furthermore, one of the glutathione S-transferases (GSTs) was demonstrated to play a critical role in the minus-strand RNA synthesis of BaMV and was also involved in anti-oxidation processes in cells (Chen et al., 2013). Therefore, relieving oxidative stress by providing antioxidants such as GST or CA might provide an optimal condition for virus replication. In other words, disturbing the appropriate environment by reducing GST or CA could rapidly affect virus replication at the early time point of infection. We found reduced BaMV accumulation in the CA-knockdown *N. benthamiana* protoplasts at 24 hpi (**Figure 2A**). If NbCA is simply an antioxidant in general, it should favor both endogenous and exogenous templates in the *in vitro* replication assays. By contrast, the coat protein accumulation of CMV and another potexvirus, PVX, did not differ from that in the control (**Figure 2**). We assumed that chloroplast-localized NbCA would affect viruses that replicate in chloroplasts. CA is involved in various biological processes including SA binding (Slaymaker et al., 2002), however, the main receptors for SA signaling are unlikely in chloroplasts (Yan and Dong, 2014). Although SA is synthesized in chloroplast, it needs to be exported to the cytoplasm to regulate immune responses (Serrano et al., 2013). The SA-mediated defense pathway might be affected by virus infection (Li et al., 2016), but not simply affected by reducing CA expression.

Another possibility for NbCA assisting BaMV is fine-tuning the condition for viral RNA replication. Because BaMV



**FIGURE 6 |** *In vitro* replication assay with the RNA templates in various pH conditions. **(A)** The relative RdRp activity of *in vitro* replication assay with endogenous and exogenous templates as indicated. The RdRp activity with pH 8.1 was set to 100%. Each point on the graph is the mean relative RdRp activity with the standard errors derived from three independent experiments. **(B)** Representative results of the *in vitro* replication assay.

replicates in chloroplasts and possibly associates with the thylakoid membrane in stroma (Cheng et al., 2013a). The pH value of the stroma is approximately 8, as the condition we have used in the *in vitro* replication assay (**Figure 6**). The replication complex associated with various host proteins including CA on the thylakoid membrane might create a replication competent microenvironment. Thus, the optimal condition for BaMV initiation and elongation could be regulated (**Figure 6**).

One of the CA activities in general is converting one carbon dioxide into bicarbonate and releasing one proton, which might act on the replicase complex and change the proton concentration at the microenvironment level to initiate RNA synthesis. Once the initiation kicks in, a switch from initiation to elongation is needed to increase pH for efficient elongation by turning off the NbCA activity or using another factor to replace NbCA. A possible candidate that could reduce the proton concentration is ferredoxin-NADP<sup>+</sup> oxidoreductase (FNR). FNR transfers electrons from the reduced form of ferredoxin (Fd) to NADP<sup>+</sup> and produces NADPH that consumes a proton with the reaction  $2 \text{Fd}_{\text{red}} + \text{NADP}^+ + \text{H}^+ \rightarrow 2 \text{Fd}_{\text{ox}} + \text{NADPH}$  (Mulo, 2011).

## CONCLUSION

We have identified a host factor that could assist in BaMV RNA replication. This factor, NbCA, could play a role in regulating the switch of initiation and elongation of RNA synthesis.

## AUTHOR CONTRIBUTIONS

I-HC and C-HT designed the research, analyzed the data and wrote the manuscript. AT, Y-PH, I-FW, and S-FC performed the experiments. Y-HH and C-HT participated in data analysis and discussion.

## FUNDING

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# Host Factors Involved in the Intracellular Movement of *Bamboo mosaic virus*

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Viruses move intracellularly to their replication compartments, and the newly synthesized viral complexes are transported to neighboring cells through hijacking of the host endomembrane systems. During these processes, numerous interactions occur among viral proteins, host proteins, and the cytoskeleton system. This review mainly focuses on the plant endomembrane network, which may be utilized by *Bamboo mosaic virus* (BaMV) to move to its replication compartment, and summarizes the host factors that may be directly involved in delivering BaMV cargoes during intracellular movement. Accumulating evidence indicates that plant endomembrane systems are highly similar but exhibit significant variations from those of other eukaryotic cells. Several *Nicotiana benthamiana* host proteins have recently been identified to participate in the intracellular movement of BaMV. Chloroplast phosphoglycerate kinase, a host protein transported to chloroplasts, binds to BaMV RNAs and facilitates BaMV replication. NbRABG3f is a small GTPase that plays an essential role in vesicle transportation and is also involved in BaMV replication. These two host proteins may deliver BaMV to the replication compartment. Rab GTPase activation protein 1, which switches Rab GTPase to the inactive conformation, participates in the cell-to-cell movement of BaMV, possibly by trafficking BaMV cargo to neighboring cells after replication. By analyzing the host factors involved in the intracellular movement of BaMV and the current knowledge of plant endomembrane systems, a tentative model for BaMV transport to its replication site within plant cells is proposed.

**Keywords:** *Bamboo mosaic virus*, intracellular movement, vesicle trafficking, endomembrane system, host factors

## INTRODUCTION

Membrane trafficking delivers materials between the endomembrane system and the plasma membrane and therefore plays an essential role in cell survival and development (Cheung and de Vries, 2008). Many animal microbes that reproduce intracellularly, including viruses and bacteria, have been shown to utilize host endomembrane trafficking and the autophagy system for intracellular transport within the host cells (Cossart and Helenius, 2014; Yamauchi and Greber, 2016), whereas other microbes alter the degradation pathway used by the host cells to destroy the intruding pathogens (Miller and Krijnse-Locker, 2008; Mudhakir and Harashima, 2009; Yamauchi and Greber, 2016). Rab proteins are small GTPases involved in membrane trafficking of cells, specifically in vesicle formation and fusion. Recent studies of *Salmonella enterica* Typhimurium,

an animal pathogenic bacterium, have identified several bacterial effectors that target various Rab proteins for replication within the host cells (Spano et al., 2011, 2016; McGourty et al., 2012; D’Costa et al., 2015).

Many plant viruses induce membrane remodeling after infecting their host cells. Studies focusing on the modification of endomembrane systems induced by plant viruses have substantially improved our understanding of the intracellular movement of plant viruses. Membrane targeting or recruitment by viral proteins in the viral replication compartment is often the cause of endomembrane remodeling, as has been demonstrated for many viruses (Wei et al., 2010, 2013; Hyodo et al., 2014; Xu and Nagy, 2014). *Grapevine fanleaf nepovirus* (GFLV) infection induces structural changes in the endoplasmic reticulum (ER) membranes to allow virus replication in the ER-derived membrane. Although host factors have not been identified, inhibitor treatment has demonstrated that vesicle transport between Golgi and ER is essential for GFLV replication (Ritzenthaler et al., 2002; Fuchs et al., 2017). A study on plant potyviruses revealed that the 6k2 viral protein of *Turnip mosaic virus* (TuMV) induces the formation of vesicles derived from ER and targets them to the chloroplasts for replication (Wei et al., 2010). *Melon necrotic spot virus* (MNSV) has been shown to replicate in mitochondria, which is significantly altered by the virus-encoded p29 protein targeting the mitochondria membrane (Mochizuki et al., 2009; Gomez-Aix et al., 2015). ER disorder has also been observed, and the p7B movement protein of MNSV is localized to ER, Golgi, actin filaments, and plasmodesmata. Disruption of the transport between ER and Golgi results in the accumulation of p7B within the ER. Therefore, the ER-to-Golgi secretory pathway could be involved in the intra- and intercellular movement of MNSV (Genoves et al., 2010).

In addition to the viral encoded proteins, host factors are also involved in the membrane remodeling process. Using yeast as a model system and by performing further testing in host plants, several studies on *Tomato bushy stunt virus* (TBSV) have demonstrated that host factors are responsible for delivering components to remodel the membrane-associated compartment for viral replication (Barajas et al., 2014; Xu and Nagy, 2016). Heat shock protein 70 is associated with the replication complex of TBSV and facilitates the insertion of viral replication proteins into the yeast membrane (Wang et al., 2009). The GTP-bound active form of Rab5-positive endosome is hijacked by TBSV for enrichment of phosphatidylethanolamine at the replication site (Xu and Nagy, 2016). A host SNARE protein, Syp71, mediates the fusion between the TuMV-induced vesicles and chloroplasts, which is required for TuMV infection (Wei et al., 2013). In yeast, membrane-shaping reticulon homology domain proteins are crucial for the formation of the replication compartment induced by the *Brome mosaic virus* (BMV) 1a protein (Diaz et al., 2010). These findings indicate that membrane trafficking and targeting are essential processes for plant virus replication.

Several pathways of intracellular movement have been proposed for animal viruses after they enter the host cells (Mudhakir and Harashima, 2009). By contrast, the trafficking pathways for the intracellular movement of plant viruses to their

replication sites within the host cells remain largely unknown. Particularly, the endomembrane trafficking systems in plants seem to be more complicated and have not been completely revealed (Saito and Ueda, 2009; Uemura, 2016). Moreover, studies of the intracellular movement of plant viruses have mostly focused on cell-to-cell movement through the plasmodesmata; these intracellular movement pathways of plant viruses have been reviewed in several articles (Park et al., 2014; Heinlein, 2015; Liou et al., 2015). Host factors participating in membrane trafficking or protein targeting may play roles in delivering BaMV or its cargoes to the replication sites. Based on the current knowledge of intracellular trafficking pathways in plants, a model for the intracellular movement of BaMV to its replication compartment is proposed. After replication, plant viruses travel intracellularly to reach plasmodesmata for cell-to-cell movement. A vesicle trafficking-related host protein participates in BaMV cell-to-cell movement; its potential role in BaMV intracellular movement is also discussed in this review.

## POSSIBLE REPLICATION COMPARTMENTS FOR BaMV

Virus infection commonly induces the formation of dynamic membrane-associated structures that are associated to the virus replication and movement (Grangeon et al., 2012; Heinlein, 2015). Chloroplasts are one of the types of compartments suitable for plant virus replication (Wei et al., 2010; Zhao et al., 2016). Using BaMV 3' RNA as a probe for *in situ* hybridization through electronic microscopy, BaMV viral RNAs were detected within several organelles of green bamboo leaf cells, including chloroplasts, mitochondria, and nuclei (Lin et al., 1993). Phage MS2 coat protein can specifically bind to its own RNA sequence, and viral genomic RNA engineered to contain the MS2 sequence can be traced within the cells through the binding of GFP-fused MS2 coat protein (Zhang and Simon, 2003). Recently, through confocal microscopy, BaMV viral RNA expressing the phage MS2 coat protein binding sequence was found to localize within chloroplasts. Furthermore, negative-strand BaMV RNA was found in the isolated chloroplasts, demonstrating that chloroplasts may be among the BaMV replication compartments within the host cells (Cheng et al., 2013a).

In contrast, *Potato virus X* (PVX), another potexvirus, has been found to replicate in the ER membrane (Doronin and Hemenway, 1996; Park et al., 2014); in addition, TGB2/3 has recently been shown to remodel the ER membrane at plasmodesmata, where PVX coupled the replication and movement to the neighboring cells (Tilsner et al., 2013).

## HOST FACTORS PROBABLY INVOLVED IN THE INTRACELLULAR TARGETING OF BaMV TO THE REPLICATION COMPARTMENT

After entering the host cells, similar to other viruses, BaMV must travel to its replication site intracellularly. Recently, several

host factors involved in BaMV replication have been identified through copurification with the BaMV replicase complex (Cheng et al., 2009; Prasantha et al., 2011; Huang et al., 2012; Lee et al., 2015), binding to the viral RNAs (Lin et al., 2007; Cheng et al., 2013a), and cDNA-amplified fragment length polymorphism (cDNA-AFLP) analysis (Chen et al., 2013; Huang et al., 2013, 2016). Among these host factors, three are possibly involved in the transportation of BaMV or its related cargoes within host cells. Chloroplast phosphoglycerate kinase (chl-PGK) (Lin et al., 2007; Cheng et al., 2013a) and NbRABG3f (Huang et al., 2016) are related to BaMV replication, whereas NbRabGAP1 is essential for the intercellular movement of BaMV (Huang et al., 2013).

In *Nicotiana benthamiana*, chl-PGK, a nuclear-encoded chloroplast protein, was found to bind to the 3' untranslated region (3' UTR) of BaMV genomic RNAs (Lin et al., 2007). Knocking down the expression or mistargeting of chl-PGK reduced BaMV accumulation in *N. benthamiana* protoplasts, indicating that chl-PGK participates in BaMV replication (Cheng et al., 2013a). Redirecting a BaMV RNA binding protein, EF1a, to chloroplasts rescued the reduction of BaMV caused by the knock-down of chl-PGK. These results demonstrate that chl-PGK likely assists in the targeting of BaMV to the chloroplasts for replication (Cheng et al., 2013a). Recently, a chl-PGK from *Arabidopsis thaliana* was identified as a requirement for efficient *Watermelon mosaic virus* (WMV) (Ouibrahim et al., 2014) and *Plum pox virus* (Poque et al., 2015) infection. Although the replication compartment of WMV is unknown, the 6K protein of other potyviruses induces the formation of mobile vesicles and transports the viruses from the ER to the chloroplast membrane for viral replication (Wei et al., 2010). Therefore, chl-PGK may be involved in the intracellular transport of different plant viruses to the chloroplasts for replication.

A recent study identified that NbRABG3f, a Rab small GTPase, is involved in BaMV replication (Huang et al., 2016). Rab small GTPases are involved in vesicle trafficking within cells. Rab proteins alternate between a GTP-bound active form and GDP-bound inactive form, and this switching is accelerated by their regulatory proteins (Cherfils and Zeghouf, 2013). GTP-bound Rab proteins bud from the donor compartment and fuse with the acceptor compartment, where GTP is hydrolyzed by GTPase-activating proteins (GAPs) (Cherfils and Zeghouf, 2013). Based on sequence comparison, NbRABG3f is homologous to animal cell Rab7 protein and to *A. thaliana* RabG3f (Huang et al., 2016). cDNA-AFLP analysis revealed that NbRABG3f expression was upregulated after BaMV inoculation (Cheng et al., 2010). The GDP-bound form of Rab GTPase can be used to trace the donor compartment of Rab proteins (Sieczkarski and Whittaker, 2002); thus, confocal microscopy revealed that the GDP-bound form mutant of NbRABG3f was localized to the Golgi compartment (Huang et al., 2016). In plant endomembrane trafficking systems, endocytotic materials are transferred to the *trans*-Golgi network for further sorting (Zhuang et al., 2015). As a plant defense mechanism pathway, intruding pathogens are likely delivered to the multi-vesicular body (MVE)/prevacuolar compartment (PVC), or autophagosome and then delivered to the vacuoles for degradation (Teh and Hofius, 2014). Successful pathogen infection results from a redirection of the pathway to their

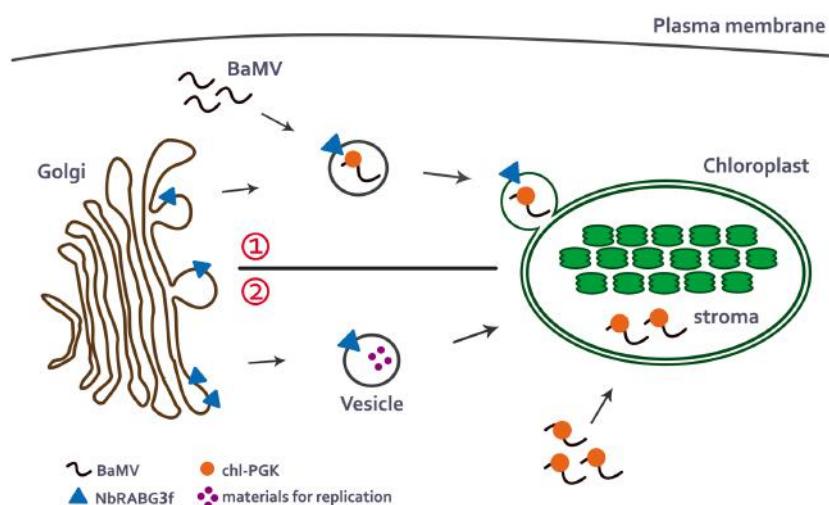
replication compartments rather than the degradation pathway (Patarroyo et al., 2012; Dong and Levine, 2013). According to the finding that NbRABG3f is derived from the Golgi compartment (Huang et al., 2016) and that chloroplasts are likely to be the replication sites of BaMV (Lin et al., 1993; Cheng et al., 2013a), BaMV may utilize NbRABG3f or NbRABG3f-associated vesicles for transport to chloroplasts. However, the acceptor membrane of NbRABG3f has not yet been identified. Knowledge of the destination of NbRABG3f in the endomembrane systems will verify whether BaMV hijacks NbRABG3f and redirects its route or instead utilizes NbRABG3f-associated vesicle and follows its pathway. Further investigation of the interaction of BaMV with NbRABG3f and other Rab proteins and further evaluation of the colocalization of BaMV and various marker proteins involved in endomembrane trafficking will reveal the intracellular transportation pathway for BaMV.

## POSSIBLE MODEL FOR BaMV INTRACELLULAR MOVEMENT TO ITS REPLICATION COMPARTMENT

In uninfected *N. benthamiana* cells, NbRABG3f and its associated vesicles are generated by the Golgi compartment (Huang et al., 2016). The destination of NbRABG3f is currently unknown. According to a putative model proposed for *Arabidopsis*, the endocytosed materials can be (1) delivered to the MVE/PVC, or autophagosome and then transported to the vacuoles for degradation, or (2) recycled into the plasma membrane (Uemura, 2016; Vukasinovic and Zarsky, 2016). A previous study demonstrated that *Arabidopsis* AtRABG3f mediates transport from PVC to the vacuole (Cui et al., 2014). Although the donor membrane of NbRABG3f is different from that of AtRABG3f, possibly because of variation in the C-terminal sequence, based on the fact that NbRABG3f is highly homologous to AtRABG3f (Huang et al., 2016), NbRABG3f likely participates in the transport of vesicles to other endomembrane systems rather than in their delivery to the recycling pathway.

According to current knowledge of the intracellular trafficking pathways in plants and studies of NbRABG3f and chl-PGK, BaMV may utilize NbRABG3f-associated vesicles for vesicle trafficking. During vesicle trafficking, chl-PGK is recruited and assists in the targeting of the BaMV complex to the chloroplasts, which are one of the types of BaMV replication compartments (Cheng et al., 2013a) (**Figure 1**, upper). By contrast, similar to the role of Rab5 protein in TBSV replication (Xu and Nagy, 2016), NbRABG3f may deliver the materials required for BaMV replication to the chloroplasts, and chl-PGK may bind BaMV RNA and direct the BaMV complex to the chloroplast for replication (**Figure 1**, lower).

Although the transportation pathway for chloroplast-targeting proteins to the chloroplast is not completely clear, to date, several pathways have been proposed for proteins transported to the chloroplasts after translation (Radhamony and Theg, 2006; Shi and Theg, 2013). Most chloroplast proteins contain an N-terminal cleavable transit peptide that mediates the



**FIGURE 1 | A possible model for BaMV intracellular movement to the chloroplasts.** In the normal membrane-trafficking pathway, NbRABG3f-associated vesicles bud from Golgi compartment. There are two possible pathways that NbRABG3f and chl-PGK may participate in delivering of BaMV cargoes to the chloroplasts. (1) When BaMV infects the cells, it may hijack the NbRABG3f-associated vesicles and redirect the vesicles to the chloroplast which is one of its replication compartments. During the transportation to the chloroplasts, chl-PGK is recruited to the BaMV complex and targets BaMV complex to the chloroplasts stroma (**Upper**). (2) Alternatively, NbRABG3f-associated vesicles may deliver materials required for BaMV replication to the chloroplasts and chl-PGK binds to BaMV viral RNA and targets it to the chloroplast stroma for replication (**Lower**).

targeting of the protein to the chloroplasts (Shi and Theg, 2013). *N. benthamiana* chl-PGK also contains a putative N-terminal transit peptide (Cheng et al., 2013a), indicating that it can be transported from the ER to the chloroplast stroma, and that such transport is directed by the N-terminal transit peptide. Whether chl-PGK is recruited to the NbRABG3f-associated vesicle or these two host proteins are involved in separate steps of BaMV transportation is an interesting question that remains to be explored. Future studies should investigate whether chl-PGK interacts with NbRABG3f or NbRABG3f-associated vesicles during the transportation process.

## A HOST FACTOR POSSIBLY INVOLVED IN BaMV VESICLE TRAFFICKING TO THE PLASMODESMATA FOR INTERCELLULAR MOVEMENT

After the replication of plant viruses, virion or virus ribonucleoprotein (vRNP) facilitates their intercellular movement through the plasmodesmata. Unlike PVX, which moves intercellularly as vRNP, BaMV is likely to move as a virion associated with TGBp2 and TGBp3-based ER membrane (Chou et al., 2013). Several BaMV viral proteins and *N. benthamiana* host factors have been demonstrated to facilitate the intercellular movement, but not the replication, of BaMV (Cheng et al., 2013b; Huang et al., 2013; Hung et al., 2014). A recent, thorough review of the intercellular movement of BaMV hypothesizes the possible roles of the viral and host proteins in the intercellular movement of BaMV (Liou et al., 2015). Among the host proteins involved in the intercellular movement of BaMV, NbRabGAP1,

a Rab-GTPase activation protein, has been identified through cDNA-AFLP analysis, and its expression is upregulated after BaMV inoculation (Huang et al., 2013). RabGAP contains the TBC (Tre2/Bub2/Cdc16) catalytic domain that can promote GTP hydrolysis and thus inactivates Rab proteins (Frasa et al., 2012). Rab proteins participate in the regulation of vesicle formation and trafficking in the endomembrane system. In the study of NbRabGAP1, low NbRabGAP1 expression reduced BaMV accumulation in *N. benthamiana* leaves, but not in protoplasts, whereas overexpression of NbRabGAP1 exerted the opposite effect. Based on these results, it is hypothesized that NbRabGAP1 is involved in the delivery of the BaMV/BaMV-related cargo from the virus replication complex to neighboring cells (Huang et al., 2013; Liou et al., 2015). An attempt to examine the interaction between NbRabGAP1 and NbRABG3f was unsuccessful (unpublished data). This outcome was expected because NbRabG3f participates in BaMV replication, whereas NbRabGAP1 assists in the intercellular movement of BaMV. Similar to PVX, the BaMV movement proteins TGBp2 and TGBp3 are ER-targeting membrane proteins (Hsu et al., 2008). The BaMV infectious complex has been proposed to move from perinuclear ER-derived membrane-bound bodies (MBB) to the plasmodesmata (Wu et al., 2011; Liou et al., 2015). After BaMV replication, NbRabGAP1 possibly participates in the delivery of BaMV cargoes with an unknown Rab protein toward the plasmodesmata through ER and post-ER secretory pathways. Alternatively, NbRabGAP1 may recycle the BaMV movement proteins from plasmodesmata to assist in the next round of BaMV transportation (Huang et al., 2013). However, the connection between chloroplasts and the MBB requires further investigation to unveil the intracellular route after BaMV replication.

Studies in PVX have indicated that its TGB2/3 movement proteins induce ER-derived granular vesicles, which are essential for PVX cell-to-cell movement (Ju et al., 2007); therefore, the PVX movement complex may be transported through these vesicles to plasmodesmata (Verchot-Lubicz et al., 2010). Accordingly, as yet unidentified host factors may be present that function as Rab or RabGAP proteins to facilitate PVX movement.

The kinases CK2 and NbSTKL are other host factors also involved in the cell-to-cell movement of BaMV (Cheng et al., 2013b; Hung et al., 2014). Knowledge on host factors that affect the intercellular movement of potexviruses have been reviewed recently (Park et al., 2014; Liou et al., 2015). Their functions in assisting BaMV or other potexvirus movement have also been discussed in a recent review, which reported that they might not directly participate in the transportation of BaMV cargoes (Liou et al., 2015). Therefore, they are not included in this review.

## SUMMARY

In this review, I summarized the host factors that participate in membrane trafficking and a specific chloroplast targeting protein that may participate in BaMV transportation within the cells. A possible model was proposed to demonstrate how BaMV is delivered to its replication compartment. One of the host factors, NbRABG3f, is a small GTPase that mediates vesicle trafficking and is derived from the Golgi compartment. Both GTPase activity and membrane-targeting ability are essential for BaMV replication. Another host protein, chl-PGK, is a

chloroplast-targeting protein, and its targeting ability is required for BaMV replication. Therefore, NbRABG3f and chl-PGK may play roles in delivering BaMV and its related complex to chloroplasts, which are a type of BaMV replication compartment. After completing replication, BaMV cargoes are delivered to the plasmodesmata for intercellular movement. NbRabGAP1, a Rab-associated protein, is not involved in BaMV replication but participates in its intercellular movement. Accordingly, NbRabGAP1 may be involved in the intracellular transport of the BaMV complex to the plasmodesmata after BaMV replication. Additional studies on dissecting the co-localization or interaction between BaMV and other vesicle proteins are required to clarify the intracellular movement pathway for BaMV.

## AUTHOR CONTRIBUTIONS

C-PC wrote and edited the paper.

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# Interfering Satellite RNAs of *Bamboo mosaic virus*

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Satellite RNAs (satRNAs) are sub-viral agents that may interact with their cognate helper virus (HV) and host plant synergistically and/or antagonistically. SatRNAs totally depend on the HV for replication, so satRNAs and HV usually evolve similar secondary or tertiary RNA structures that are recognized by a replication complex, although satRNAs and HV do not share an appreciable sequence homology. The satRNAs of *Bamboo mosaic virus* (satBaMV), the only satRNAs of the genus *Potexvirus*, have become one of the models of how satRNAs can modulate HV replication and virus-induced symptoms. In this review, we summarize the molecular mechanisms underlying the interaction of interfering satBaMV and BaMV. Like other satRNAs, satBaMV mimics the secondary structures of 5'- and 3'-untranslated regions (UTRs) of BaMV as a molecular pretender. However, a conserved apical hairpin stem loop (AHSL) in the 5'-UTR of satBaMV was found as the key determinant for downregulating BaMV replication. In particular, two unique nucleotides (C<sup>60</sup> and C<sup>83</sup>) in the AHSL of satBaMVs determine the satBaMV interference ability by competing for the replication machinery. Thus, transgenic plants expressing interfering satBaMV could confer resistance to BaMV, and interfering satBaMV could be used as biological-control agent. Unlike two major anti-viral mechanisms, RNA silencing and salicylic acid-mediated immunity, our findings in plants by *in vivo* competition assay and RNA deep sequencing suggested replication competition is involved in this transgenic satBaMV-mediated BaMV interference. We propose how a single nucleotide of satBaMV can make a great change in BaMV pathogenicity and the underlying mechanism.

**Keywords:** interfering, satellite RNA, BaMV, competition, RNA silencing

## INTRODUCTION

Satellite RNAs (satRNAs) are short RNA molecules that share no or little sequence homology to their cognate helper virus (HV) but totally depend on the HV for replication, encapsidation and efficient movement (Hu et al., 2009; Briddon et al., 2012). The homology sequence between satRNAs and their HVs often resides at the 5' and 3' regions. Usually conserved secondary structure functions such as the *cis*-acting element are essential for replicase recognition acting as mimicry of molecular pretenders at the 5' and 3' regions. SatRNA mimicry is mostly conserved in higher-order RNA structures. As well, satRNAs may adopt different mimicry at different stages of virus infection such as replication and translation (Huang et al., 2010).

Satellite RNAs have attracted great interest in the past decades because they can modulate symptoms caused by their HVs (Palukaitis, 1988; Li and Simon, 1990; Collmer and Howell, 1992; Hsu et al., 1998), alter HV RNA accumulation (Buzayan et al., 1986; Gal-On et al., 1995;

Hsu et al., 1998), enhance HV movement (Zhang and Simon, 2003; Simon et al., 2004) and in at least one case, affect the infection cycle of their HV, for example, during insect transmission (Robinson et al., 1999; Taliinsky et al., 2000). One of the most fascinating characteristics of satRNAs is their interference ability. There are many cases of symptom-attenuating satRNAs, such as satRNAs of isolates of the species *Bamboo mosaic virus* (BaMV), *Cucumber mosaic virus* (CMV), *Peanut stunt virus* (PSV), *Grapevine fanleaf virus*, *Artichoke mottled crinkle virus*, *Cymbidium ringspot virus* (CymRSV), *Tobacco ringspot virus* (TobRSV), and *Groundnut rosette virus* (GRV) (Roossinck et al., 1992; Simon et al., 2004).

Satellite RNAs of BaMV (satBaMVs) are well studied. Natural isolates of satBaMVs have been collected from BaMV-infected symptomatic bamboo plants worldwide to analyze the genetic evolution and phylogeny of satBaMVs (Liu et al., 1997; Wang et al., 2014). The mimicry of satBaMVs among the 5'- and 3'-untranslated regions (UTRs) have been investigated thoroughly (Annamalai et al., 2003; Huang et al., 2009), and the biological function of satBaMV-encoded protein elucidated its role in satBaMV replication (Lin et al., 1996), movement (Vijaypalani et al., 2006, 2012; Chang et al., 2016) and interference in BaMV replication (Hsu et al., 2006).

In this review, we focus on studies of interfering satBaMVs and a possible mechanism of satBaMVs interfering in BaMV infection.

## BAMV AND ITS ASSOCIATED SATBAMVS

*Bamboo mosaic virus* is a single-stranded positive-sense RNA virus containing five open reading frames (ORFs) that belongs to the genus *Potexvirus* of the family *Alphaflexiviridae* (Lin et al., 1994). ORF1 encodes a replicase-related protein with three functional domains for BaMV replication: methyltransferase (Li et al., 2001a; Huang et al., 2004), helicase (Li et al., 2001b) and RNA-dependent RNA polymerase (RdRp) (Li et al., 1998). ORF2 to four encode triple gene block proteins, which are three overlapping proteins essential for BaMV movement (Wung et al., 1999; Lin et al., 2004, 2006). ORF5 encodes a coat protein (CP) for BaMV encapsidation, movement (Lee et al., 2011) and symptom formation (Lan et al., 2010) (**Figure 1A**).

*Bamboo mosaic virus* causes mosaic symptoms on infected bamboo leaves and infects at least 13 economically important bamboo species in Taiwan (Lin et al., 1993). In BaMV-infected bamboo, small single-stranded positive-sense RNA molecules that share no sequence homology with BaMV but replicate and encapsidate associated BaMV are defined as satBaMVs (Lin and Hsu, 1994). SatBaMV is the only potexvirus-associated satRNA. It is a 836-nt linear RNA molecule that encodes a 20-kDa non-structural protein (P20) flanked by a 159-nt 5'-UTR and 125-nt 3'-UTR (Lin and Hsu, 1994; **Figure 1A**). P20 is not essential for satBaMV replication (Lin et al., 1996), but it preferentially binds to satBaMV RNA (Tsai et al., 1999). However, P20 is necessary for satBaMV long-distance transport in BaMV-co-infected *Nicotiana benthamiana* (Vijaypalani et al., 2006, 2012; Chang et al., 2016). In the absence of BaMV, satBaMV RNA

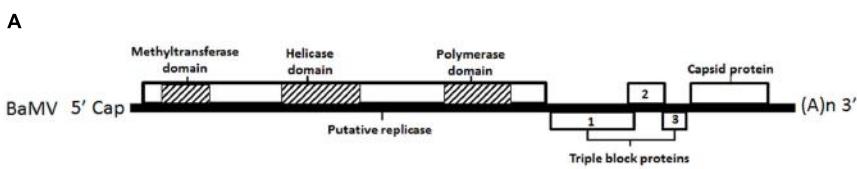
could undergo autonomous long-distance movement *in planta* (Chang et al., 2016).

Three phylogenetic satBaMV groups were classified from natural satBaMV isolates derived from 10 infected bamboo species in different locations of Taiwan, Hainan Island of China and Delhi, India (Liu et al., 1997; Yeh et al., 2004; Wang et al., 2014). Clade I contains all other satBaMVs except most of those isolated from Ma bamboo (*Dendrocalamus latiflorus* Munro) and all populations from *Bambusa vulgaris*. All satBaMVs in clades II and III are derived almost entirely from Ma bamboo from the Taipei Botanical Garden in Taiwan and *B. vulgaris* in India, respectively (Wang et al., 2014).

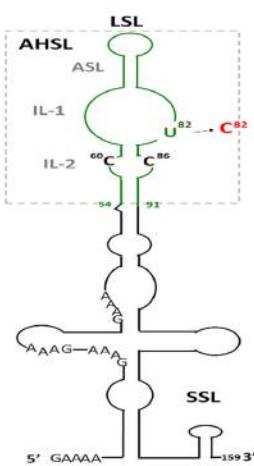
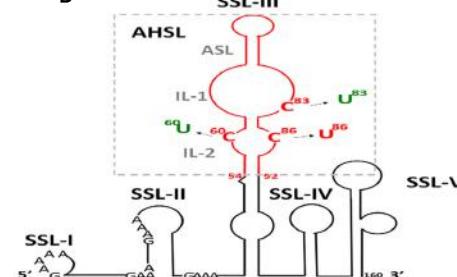
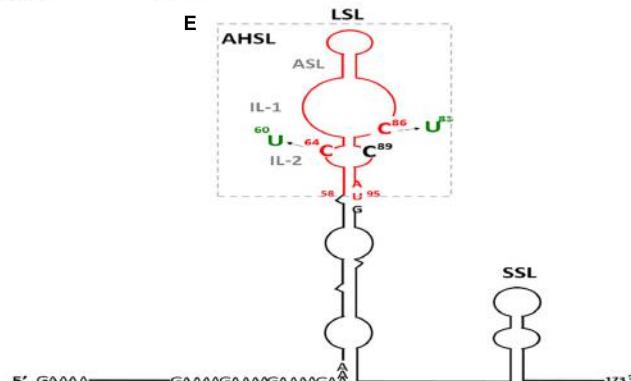
Sequence analysis of satBaMV isolates showed a hypervariable region with the greatest sequence variation in the satBaMV 5'-UTR but a conserved secondary RNA structure (Yeh et al., 2004). SatBaMV is totally dependent on BaMV for replication and encapsidation (Lin and Hsu, 1994). Therefore, 5'- and 3'-UTRs of satBaMV evolved similar RNA secondary structures and functional RNA elements with BaMV to recruit the RdRp encoded by BaMV for replication. These features include GAAA(A) repeats at the 5'-UTR and conserved hexanucleotides (ACCUAA) and polyadenylation signals (AAUAAA) at the 3'-UTR (Lin and Hsu, 1994; Lin et al., 1994). As well, the secondary structures of the satBaMV 3'-UTR contain two small stem-loops (SLA and SLB) and one large stem-loop (SLC) that are similar to the domains B, C, and D of the BaMV 3'-UTR, respectively (Cheng and Tsai, 1999; Huang et al., 2009). One of the alluring properties of satBaMVs is that some natural satBaMV isolates feature antagonistic ability against BaMV replication (Hsu et al., 1998, 2006). However, interfering satBaMVs isolated from different bamboo species and locations are not grouped in the same phylogenetic clades (Yeh et al., 2004). The mechanisms underlying satRNA-mediated HV interference is fascinating, but most cases have not been clearly demonstrated.

## THE DETERMINANT OF SATBAMV INTERFERENCE RESIDES IN THE 5'-UTR APICAL HAIRPIN STEM LOOP (AHSL)

Two satBaMV isolates, BSF4 and BSL6, exhibit different phenotypes in *N. benthamiana* co-infected with BaMV and satBaMV (Hsu et al., 1998). Attenuated BaMV-induced symptoms were found associated with reduced BaMV level (Hsu et al., 1998). The sequence of the BSF4 and BSL6 5'-UTR shares 92% identity, with only 13 mismatches (**Figure 1B**), but the secondary structures greatly differed, as revealed by enzymatic probing with RNases A, T1, T2, and V1. The secondary structures of the non-interfering BSF4 5'-UTR contain a large stem loop (LSL) and a small stem loop (SSL) (**Figure 1C**; Annamalai et al., 2003), whereas the interfering BSL6 5'-UTR contains five SSLs (**Figure 1D**; Chen et al., 2007). However, the 5'-UTR hypervariable region of both BSF4 (in LSL) and BSL6 (SSL-III) features a conserved apical hairpin stem loop (AHSL) structure including two internal loops (ILs; IL-1 and IL-2) (**Figures 1C,D**; Annamalai et al., 2003; Chen et al., 2007). *In silico* secondary structure prediction of the 5'-UTR of natural satBaMV isolates

**B**

|      |   |     |
|------|---|-----|
| BaMV | GAAAACCACCTCC-AAACGAAACGAAAAGAAA--TTAAAAGACGAAGAACAACTGCCATT          | 60  |
| BSF4 | *****T*A**GC*****CA**TCG**C*G*A*CACTA-***C-*-*AGGG--                  | 56  |
| BSL6 | *****T*A**GC*****CA**TCG**C*G*A*TACTT-***C-*-*AGGG--                  | 56  |
|      |   |     |
| BaMV | GTCCCCCTACAATCGCCC-TGCGCGTGC GGCAACAA <b>TGGCACTCGTTCTAAAGTCTTG</b> A | 119 |
| BSF4 | <b>CC*****T*G**CCGC-**AGG***T*****GGCCCC*TG-GATAGG***C-----*T</b>     | 110 |
| BSL6 | <b>GC*****T*G**TGCTT***GCG***C***GCCCC*TG-GATAGG***C-----*A</b>       | 111 |
|      |   |     |
| BaMV | CAGCATCACCGACCCATCCTT-GAGGGCTGTACTCCAAGAGGAAGCGCACTCCCA               | 173 |
| BSF4 | -G*TG*TC**CG*A*-***G*C***C*G*-**A*A*G*CGCTT*C*AAGACG----              | 159 |
| BSL6 | -G*TA*TC**CG*A*-***G*C***C*G*-**A*A*G*CGCTT*C*AAGACG----              | 160 |

**C****D****E**

**FIGURE 1 | Genome map of satellite *Bamboo mosaic virus* (satBaMV) and BaMV (A), sequence alignment of BaMV, BSF4, and BSL6 5'-UTR (B) and secondary structures of 5'-UTR of satBaMV, BSF4 (C) and BSL6 (D) and 5'-termini of BaMV (E) and their derived mutants. \*indicates identical nucleotide. Different nucleotides between BSF4 and BSL6 sequence are marked by gray shade. The apical hairpin stem loop (AHSL) structures of satBaMV and BaMV are boxed, and all contain an apical stem loop (ASL) and two internal loops (IL-1 and IL-2). The common GAAA(A) repeats in the 5'-UTRs are indicated. The AUG sequence indicates the start codon of the BaMV open reading frame 1 (ORF1). Green and red indicate the non-interfering and interfering type. LSL, large stem loop; SSL, small stem loop.**

by MFOLD revealed that most of the analyzed satBaMV isolates retained an identical AHSL structure despite their grouping into different phylogenetic clades (Yeh et al., 2004). Moreover, the RNA sequence in the AHSL region of BSF4 and BSL6 is interchangeable, and chimeric satBaMVs can replicate to a similar level as BSF4 and BSL6 when co-infected with BaMV in *N. benthamiana* protoplasts, so maintaining a conserved AHSL structure but not the sequence itself is essential for satBaMV replication (Yeh et al., 2004).

To elucidate the determinant of BSL6 interference of both BaMV-induced symptoms and BaMV level, chimeric satBaMV mutants with different combinations of BSF4 and BSL6 between the 5'-UTR, most coding regions of P20 and the 3'-UTR were investigated. All mutants containing the 5'-UTR of BSL6 could reduce BaMV level in both positive (+) and negative (-) strands without altering satBaMV level in *N. benthamiana* protoplasts and caused symptomless infection in *N. benthamiana* plants (Hsu et al., 2006). Moreover, both a BSL6 mutant expressing the truncated form of P20 and a frameshift mutant could reduce BaMV level, so P20 is not required for BSL6-mediated BaMV interference (Hsu et al., 2006). Furthermore, the BSL6 5' UTR alone was sufficient to interfere with (+)- and (-)-strand BaMV level and BaMV-caused symptoms when expressed in a BaMV vector driven by a sub-genomic promoter in *Chenopodium quinoa* (Hsu et al., 2006). Thus, the BSL6 5'-UTR is the determinant of the interference in BaMV replication and the interference is independent of P20 translation.

On further analyzing the RNA secondary structure of natural satBaMV isolates, an identical AHSL structure was found shared by all natural interfering satBaMVs. SatBaMV mutants that swap the AHSL region of BSF4 and BSL6 revealed that the AHSL in the 5'-UTR is the determinant of satBaMV-mediated BaMV interference (Hsu et al., 2006). To further clarify whether the structure or sequence of AHSL is more important for BSL6-mediated interference, BSL6-derived mutants with disrupted AHSL structure or only sequence substitution were used to test BaMV interference. On co-inoculation with BaMV, all mutants with disrupted AHSL structure lost the ability to reduce BaMV level. Moreover, an identical AHSL structure with the sequence (<sup>81</sup>UGC<sup>83</sup>) in IL-1 was found in all natural interfering satBaMVs, whereas a less-conserved AHSL structure or identical AHSL structure but with different sequence (<sup>81</sup>UGU<sup>83</sup>) in IL-1 was found in non-interfering satBaMVs (Chen et al., 2007). Further analysis revealed that only one nucleotide substitution in U<sup>82</sup> to C<sup>82</sup> or C<sup>83</sup> to U<sup>83</sup> of BSF4 or BSL6, respectively, could change the phenotype (Chen et al., 2007). Another nucleotide C<sup>60</sup> in IL-2 was also essential for BSL6-mediated interference. BSL6 C<sup>60</sup>U no longer reduced BaMV level (Chen et al., 2007). Thus, both the AHSL structure and two nucleotides C<sup>60</sup> in IL-2 and C<sup>83</sup> in IL-1 are essential for BSL6-mediated BaMV interference (Figures 1C,D).

Different hosts also feature a one-nucleotide substitution altering satRNA-induced symptoms or their ability to modulate HV-induced symptoms. With CMV in tomato, C<sup>215</sup>, C<sup>286</sup> and A<sup>330</sup> of WLM2-satCMV could independently affect necrosis induction with different CMV strains (Wu and Kaper, 1992; Sleat et al., 1994), and the satCMV Y-strain nucleotide 185/186 caused

yellow mosaic symptoms in tobacco (Jaegle et al., 1990). For the PSV system, U<sup>226</sup> and C<sup>262</sup> determine symptom attenuation of PSV G-satRNA in tobacco (Naidu et al., 1992). These examples all imply that the pathogenicity of satRNAs result from the complex interaction between the host, HV and satRNAs.

However, only an approximate idea was proposed for the altered RNA secondary or tertiary structure being essential for necrosis induction of WLM2-satCMV caused by a single nucleotide change (Sleat et al., 1994). How a single nucleotide of satBaMV results in such a great change in the interference of BaMV-induced symptoms and BaMV replication is a fascinating mystery that remains to be solved.

## CONSERVED SECONDARY STRUCTURES IN THE 5'-UTR OF BAMV AND SATBAMV ARE INVOLVED IN COMPETITION FOR REPLICATION COMPLEXES

Because of the HV RdRp-dependent replication of satRNAs, competition for viral RdRp between satRNAs and HV was the first hypothesized and demonstrated as a mechanism for CMV and satCMV (Wu and Kaper, 1995). However, the authors used *in vitro* replication assay, which may not reflect the complex interaction between CMV and satCMV in co-infected plants (Wu and Kaper, 1995). Although many satRNAs reduce HV accumulation, no further studies have implied RdRp competition as the determinants of satRNAs-mediated interference. In contrast, a more complete analysis of the conserved secondary structure of BaMV and satBaMV implied that replication complex competition could be the major mechanism of satBaMV-reduced BaMV level.

First, interfering satBaMV is dominant among progeny populations in protoplasts with mixed-infected BaMV and non-interfering satBaMV (Chen et al., 2012). In addition, an *in vivo* replication system revealed that the replication efficiency is higher for BSL6 than BSF4 when the two are individually supported by abundant BaMV ORF1-encoded RdRp for replication in *N. benthamiana* protoplasts (Chen et al., 2012). Hence, replication is more competent with interfering satBaMV than BaMV and non-interfering satBaMV.

Both BaMV and satBaMV depend on BaMV RdRp for replication, so whether BaMV contains a similar AHSL structure in the 5'-UTR is of interest. The 5'-UTR RNA secondary structures of all natural BaMV isolates were analyzed by MFOLD but showed no secondary structure because of a highly repetitive sequence. The conserved AHSL in LSL was found only when the sequence extended to the ORF1 region (1-173 nt) (Figure 1E; Chen et al., 2012). This secondary structure of BaMV-S was confirmed by enzyme probing (Chen et al., 2010). As predicted, all analyzed BaMV isolates showed an identical AHSL structure with C<sup>86</sup> in IL-1 and C<sup>64</sup> in IL-2 regardless of whether satBaMV was associated with their replication or whether the associated satBaMV was interfering or non-interfering (Chen et al., 2012). The C<sup>60</sup> in IL-2 and C<sup>83</sup> in IL-1 of BSL6

(corresponding nucleotide C<sup>86</sup> in IL-1 and C<sup>64</sup> in IL-2 of BaMV) are essential for satBaMV-mediated BaMV interference (**Figure 1D**; Chen et al., 2007) and also important for BaMV replication (Chen et al., 2012). The BaMV-C<sup>86</sup>U mutant lost replication ability in *N. benthamiana* protoplasts and *C. quinoa*. The replication efficiency was reduced with BaMV-C<sup>64</sup>U-mutant infection as compared with BaMV infection alone. Thus, BaMV C<sup>86</sup> is essential and C<sup>64</sup> is important for BaMV replication. Furthermore, non-interfering satBaMV BSF4 could reduce the number of local lesions and BaMV-C<sup>64</sup>U level on co-infection in *C. quinoa*, so BSF4 may be more competent than BaMV-C<sup>64</sup>U for replication. In addition, increased level of BaMV was associated with reduced BSL6 level in the mixed inoculum (Chen et al., 2012). These results demonstrate that satBaMVs interfere with BaMV replication in a dose-dependent manner via replication complex competition.

## TRILATERAL INTERACTION AMONG BAMV, SATBAMV AND HOST PLANTS: POSSIBLE INVOLVEMENT OF RNA SILENCING IN SATBAMV-MEDIATED BAMV INTERFERENCE

Interfering satRNAs attenuating HV-caused symptoms and reducing the HV level are complex interactions between the host plant, HV and satRNAs. However, the model of competition for replication complexes explains the interaction between only the HV and satRNAs. No other mechanisms were proposed and proven until large studies of RNA silencing and the generation of a large amount of next-generation sequence data from virus-infected samples. These “big” data reveal the trilateral interactions of host, HV and satRNAs. For example, small RNAs (sRNAs) of satRNAs (sat-sRNAs) can target HV and induce silencing of HV for CMV (Zhu et al., 2011); the satCMV of SD-CMV can reduce level of RNA-4A, which encodes the viral suppressor of RNA silencing 2b (VSR2b) protein, thereby diminishing the viral counter-defense strength by host immunity (Hou et al., 2011). In addition, Y satRNAs (Y-sat) of CMV can interfere in the function of VSRs by saturating the sRNA binding capacity of VSR (Shen et al., 2015). All this evidence shows that satRNAs take advantage of the host defense system and RNA silencing to interfere in HV replication.

RNA silencing is the major antiviral defense mechanism operating in a sequence-specific manner in plants (Ding, 2010). In general, double-stranded RNA formed during virus replication or the highly structured viral RNA can trigger RNA silencing by recognizing and dicing into 20- to 24-nt viral sRNAs (vsRNAs) by RNase III-like proteins, Dicer-like (DCLs) (Blevins et al., 2006). These vsRNAs are then recruited by ARGONAUTE proteins (AGO) (Mallory and Vaucheret, 2010) and target the viral RNA or host genes with a complementary sequence. The viral RNAs or target genes would be cleaved and silenced by vsRNAs via RNA degradation. However, viruses also evolve to have the counter-defense mechanism by encoding a VSR. VSRs suppress RNA silencing by four major mechanisms. The most straight-forward

and common way is by binding sRNAs. Second, they prevent the recognition and dicing of viral RNA by inhibiting DCLs. Third, they prevent the assembly of the RNA-induced silencing complex by targeting its components, such as AGOs. Finally, they inhibit the amplification of antiviral signals by interacting with RdRp or its interacting complexes (Burgyan and Havelda, 2011).

Satellite RNAs are both inducers and targets of RNA silencing. Highly structured satRNAs or satRNA-replication intermediate double-stranded RNAs induce RNA silencing and produce sat-sRNAs (Du et al., 2007; Lin et al., 2010). These sat-sRNAs can direct RNA cleavage of host genes (Shimura et al., 2011; Smith et al., 2011) or the HV genome (Zhu et al., 2011) and cause DNA methylation of host genes (Wang et al., 2001). However, unlike the HV, no satRNA encoded proteins were reported as VSRs. How interfering satBaMV manipulates the host RNA silencing immune system to reduce HV replication remains largely unknown, although strategies mediated by different satCMVs have been reported (Moriones et al., 1992; Hou et al., 2011; Shen et al., 2015). From small-RNA sequencing data, BaMV-derived sRNA (BaMV-sRNA) levels were not increased in BaMV and BSL6 co-infected samples, and no specific satBaMV-sRNAs of BSL6 could target BaMV genome (Lin et al., 2010). Although the 5'-UTR of BaMV contains a stretch of homologous sequence from nucleotides 1 to 30 (**Figure 1B**), BaMV-sRNAs and satBaMV-sRNAs of BSF4 and BSL6 generated from this region are extremely low in number (Lin et al., 2010). The sRNA hotspots within the 5'-UTR of BaMV and BSF4 located in the region from nucleotides 80 to 120 formed SLB and SLC and one strand of the stem region of SLC (Lin et al., 2010). Hence, RNA silencing may not be directly involved in satBaMV-mediated reduction in BaMV infection.

## APPLICATION OF INTERFERING SATBAMV IN BAMV RESISTANCE

*Bamboo mosaic virus* infects more than 90% of bamboo plants with pachymorph rhizomes in Taiwan, which results in great economic loss (Lin and Chen, 1991; Lin et al., 1993). Because bamboo is usually vegetatively propagated, the use of indexed, non-infected bamboo generated from meristem tip culture as propagation materials would greatly improve BaMV disease control (Hsu et al., 2000). However, BaMV spread may be through unknown vectors, mechanical injury or contaminated tools used for propagation or harvesting. How to eliminate BaMV infection in healthy plants in the field is critical. One of the promising strategies is the use of virus-resistant cultivars.

Because satRNAs can attenuate HV-induced symptoms and/or reduce HV replication, they are good candidates as biological-control agents. In the late 1980s, satCMV transgenic plants showing CMV resistance were established despite the underlying mechanism remaining unknown (Harrison et al., 1987). Interfering satBaMV could attenuate symptoms and reduce the BaMV level in co-infected plants. Thus, transgenic plants expressing interfering satBaMV would be a feasible approach to alleviate infection with BaMV. In transgenic

*N. benthamiana* expressing BSL6 satBaMV, two phenotypes were observed after BaMV infection: one group showed mild BaMV symptoms, and another group was symptomless (Lin et al., 2013). Moreover, BSL6 transgenic plants were resistant to both BaMV viral-RNA and virion infection and with better resistance to BaMV viral RNA than virion. The transgene, BSL6 replicon, was expressed at a relatively low level in transgenic lines but was highly induced after BaMV infection. Thus, highly inducing the transgene only after BaMV infection could avoid the highly expressed transgene-induced silencing in plant growth and development. Moreover, BSL6-transgenic plants are highly resistant whether under attack by BaMV viral RNA or virions. With all these features, interfering satBaMV-transgenic plants may be a good option for BaMV disease control.

RNA silencing may not be involved in the mechanism of satBaMV-mediated BaMV resistance in transgenic plants. Moreover, the plant innate immune system involving salicylic acid and jasmonic acid pathways was also not enhanced in satBaMV-transgenic plants. However, the resistance of satBaMV transgenic plants to BaMV was associated with the transgene expression level in transgenic lines under the mock condition. Non-replication satBaMV transgenic plants could not reduce BaMV replication (Lin et al., 2013). Thus, competition for replication complexes with BaMV is the possible mechanism in BaMV-resistant transgenic plants expressing interfering satBaMV.

## PERSPECTIVES

The AHSL secondary structure and two unique nucleotides ( $C^{60}$  and  $C^{83}$ ) of satBaMV 5'-UTR are critical for the interfering satBaMV reducing BaMV level and infection in plants. This AHSL structure and the critical nucleotide C in IL-1 is conserved in the BaMV 5'-UTR and also important for replication. Moreover, interfering satBaMV dose-dependently reduces BaMV level. Thus, interfering satBaMV-reduced BaMV level competes for the replication complex.

How a single nucleotide determines the interference ability of satBaMV deserves further investigation. Here we propose the possible underlying mechanism.

## Long-Distance RNA–RNA interaction

Viral RNAs are four-dimensional because of the complex tertiary interactions with the host and viral factors in specific viral infection stages. These long-distance RNA–RNA interactions control virus replication, translation and sub-genomic RNA transcription (Miller and White, 2006). Whether  $C^{60}$  in IL-2 and  $C^{83}$  in IL-1 interact with a terminal or internal element of satBaMV or BaMV critical for BaMV interference remains unknown. However, a BaMV chimeric mutant expressing the BSL6 5'-UTR driven by a sub-genomic promoter is sufficient to reduce both (+) genomic and sub-genomic RNA level without affecting (-) sub-genomic RNA level. As well, the reduced (+) genomic RNA level is greater than the (+) sub-genomic RNA level (Hsu et al., 2006). This result may imply that possible long-distance RNA–RNA interaction of satRNAs and BaMV affects

only activation or assembly of an RdRp complex competent for (+)- but not (-)-strand synthesis.

## RNA methylation

Another hypothesis for a single nucleotide of satBaMV causing a great change in interference in BaMV-induced symptoms and BaMV replication is methylation of this specific nucleotide. Ribonucleotides are ubiquitously methylated in life at nitrogen, the oxygen of the 2'OH moiety at fifth-position carbon atoms in pyrimidine, and second- and eighth-position carbon atoms in adenosines (Motorin and Helm, 2011). Methylated cytosine ( $m^5C$ ) is the most privileged. Cytosine can be easily transformed into uracil via deamination. However,  $m^5C$  cannot be converted to uracil. Cellular RNAs containing  $m^5C$  include transfer RNA (tRNA), ribosomal RNA, mRNA and non-coding RNA in both eukaryotes and prokaryotes (Squires et al., 2012; Edelheit et al., 2013; Hussain et al., 2013; Burgess et al., 2015; Delatte et al., 2016). Also,  $m^5C$  was found in some animal viruses (Dubin and Stollar, 1975; Sommer et al., 1976).  $M^5C$  is important for stabilization and  $Mg^{2+}$  binding of tRNA (Basti et al., 1996; Stuart et al., 2003; Helm, 2006), translation of mRNA (Strobel and Abelson, 1986) and weakening stimuli to the human innate immune system (Kariko et al., 2005). In adenovirus-infected HeLa cells,  $m^5C$  was found only in adenovirus RNA (Sommer et al., 1976) but not mRNA (Furuichi et al., 1975; Salditt-Georgieff et al., 1976). As well, the tRNA-like structure of an isolate of *Turnip yellow mosaic virus* injected into *Xenopus* oocytes could be methylated at cytosine (Brule et al., 1998).

How viral RNAs are specifically methylated and the biological function of  $m^5C$  in viral RNA needs further study. Here, we propose two hypotheses. One is that  $m^5C^{60}$  and  $m^5C^{83}$  may appropriately and efficiently dock into the active site of key factors of replication complexes. Alternatively, the methylation of cytosine in the tRNA-like structure of BaMV 3'-UTR may be critical for the interaction between replication complexes, BaMV 5'-UTR and 3'-UTR, and this interaction may be affected by the interfering satBaMV 5'-UTR during replication, thus reducing BaMV replication at both the (+)- and (-)-strand level. Bisulfite sequencing (Schaefer et al., 2009) could be used to elucidate whether  $C^{60}$  and  $C^{83}$  of satBaMV and C of BaMV 3'-UTR are methylated or not. However, the biological function of these methylated satBaMVs on BaMV replication is difficult to prove. A putative methyltransferase was found to interact with BaMV RdRp and suppress BaMV replication (Cheng et al., 2009). The involvement of RNA  $m^5C$  methyltransferases in satBaMV-mediated BaMV interference is worthy of further investigation.

## Host factors or miRNAs involved

Whether specific host factors are recruited by interfering satBaMV for interference remains unknown but could be tested by comparing the protein profiles bound to the 5'-UTR of BSF4 and BSL6. The specific AHSL-interacting proteins can be detected by using the 5'-UTR of BSF4 and BSL6 as probes, followed by mass spectrometry identification.

Moreover, sRNA sequencing and array analysis revealed that the plant innate immune system is not involved and RNA silencing may not be directly involved in the mechanism of satBaMV-mediated BaMV interference. However, interfering satBaMV-induced specific microRNAs (miRNAs) or specific satBaMV-sRNAs may likely target the host gene, which is important for BaMV replication or essential effectors of the host innate immune system other than RNA silencing. Thus, the involvement of RNA silencing in BSL6-mediated interference remains an open question. It could be evaluated by using plant mutants defective in key components of RNA silencing or plants overexpressing VSRs and further analyzing satBaMV-induced specific satBaMV-sRNAs, miRNAs and other types of host endogenous small RNAs. The mechanism underlying interfering satBaMV reducing BaMV level and host symptom

development remains a fascinating question requiring long-term study.

## AUTHOR CONTRIBUTIONS

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**Conflict of Interest Statement:** 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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# Antiviral Roles of Abscisic Acid in Plants

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Abscisic acid (ABA) is a key hormone involved in tuning responses to several abiotic stresses and also has remarkable impacts on plant defense against various pathogens. The roles of ABA in plant defense against bacteria and fungi are multifaceted, inducing or reducing defense responses depending on its time of action. However, ABA induces different resistance mechanisms to viruses regardless of the induction time. Recent studies have linked ABA to the antiviral silencing pathway, which interferes with virus accumulation, and the micro RNA (miRNA) pathway through which ABA affects the maturation and stability of miRNAs. ABA also induces callose deposition at plasmodesmata, a mechanism that limits viral cell-to-cell movement. Bamboo mosaic virus (BaMV) is a member of the potexvirus group and is one of the most studied viruses in terms of the effects of ABA on its accumulation and resistance. In this review, we summarize how ABA interferes with the accumulation and movement of BaMV and other viruses. We also highlight aspects of ABA that may have an effect on other types of resistance and that require further investigation.

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## INTRODUCTION

Plants adapt to or tolerate stress through production of specific hormones that are produced at very low concentrations. One of the classical and well-studied phytohormones is abscisic acid (ABA), the importance of which is highlighted by its various roles in development (such as seed dormancy, germination, and floral induction) and stress responses (such as drought, salinity, and pathogen infection) (Mauch-Mani and Mauch, 2005; Wasilewska et al., 2008; Finkelstein, 2013; Humplík et al., 2017).

Abscisic acid affects the plant defense response to pathogens of different lifestyles, such as biotrophs that thrive on a living host without killing it and necrotrophs that cause host death and thrive on dead matter (Mauch-Mani and Mauch, 2005; Fan et al., 2009; Xu et al., 2013). However, the effects of ABA are multifaceted, depending on the pathosystem studied and the timing of induction (Ton et al., 2009). ABA can enhance plant defense if it is triggered at early stages of infection by closing stomata and inducing callose deposition at cell walls (Ton et al., 2009; Ellinger et al., 2013). In contrast, if a pathogen is successfully established inside a plant tissue, then ABA induction can hamper plant defense by antagonizing other hormone pathways such as those responsible for salicylic acid (SA) or ethylene synthesis (Anderson et al., 2004; Yasuda et al., 2008).

While ABA can both induce and reduce plant defense against fungal and bacterial pathogens, it appears to only enhance plant antiviral defense as shown for several viruses (Chen et al., 2013; Alazem et al., 2014; Alazem and Lin, 2015). Two ABA-dependent defense mechanisms against viruses have been reported in plants, callose deposition at plasmodesmata (PD)

(Iglesias and Meins, 2000; De Storme and Geelen, 2014) and the RNA silencing pathway (Alazem and Lin, 2015; Alazem et al., 2017). In addition, ABA-related recessive resistance has been reported for two RNA viruses, bamboo mosaic virus (BaMV) and cucumber mosaic virus (CMV) (Alazem et al., 2014). These findings have attributed novel antiviral roles to ABA in plants, and have raised outstanding questions discussed below that require further investigations.

Bamboo mosaic virus is a positive-sense, single-stranded RNA virus of the *Potexvirus* genus (Family *Alphaflexiviridae*) with a genomic RNA of 6.4 Kb (Lin et al., 1994). BaMV genome encodes five open reading frames that translate into a replicase composed of three domains (a capping enzyme domain, a helicase-like domain, and an RNA-dependent RNA polymerase domain) (Li et al., 1998, 2001a,b; Huang et al., 2004), three movement proteins (Lin et al., 1994, 2004, 2006), and a capsid protein (Lan et al., 2010).

Since ABA effects on plant antiviral defense have been mostly studied using BaMV, here we summarize how ABA interferes with the accumulation, movement, and symptom development of BaMV and other viruses following infection. We also highlight several aspects of the ABA signaling pathway that may have potential effects on other types of antiviral resistance and that require further investigation.

## VIRUS INFECTION INDUCES ABA

Several RNA viruses have been shown to induce drought tolerance in plants, a phenomenon observed following infection by CMV, tobacco mosaic virus (TMV) and tobacco rattle virus (TRV) in different host plants including *Nicotiana tabacum*, *Beta vulgaris*, and *Oryza sativa* (Xu et al., 2008). Xu et al. (2008) ascribed this drought tolerance to the increase in the concentrations of osmoprotectants and antioxidants following viral infection. However, apart from the effects of osmolytes, drought tolerance is usually attributed to the increase of ABA content (Finkelstein, 2013). In fact, the increase of ABA content in virus-infected hosts has been reported for a number of compatible interactions (successful infection leading to disease) such as CMV/*Nicotiana benthamiana* (Alazem et al., 2014), BaMV/*Arabidopsis thaliana* and BaMV/*N. benthamiana* (Alazem et al., 2014), and TMV/*N. tabacum* (Fraser and Whennham, 1989). However, in some incompatible interactions (successful plant defense), viral infection does not induce ABA (Kovac et al., 2009; Baebler et al., 2014). For example, infection by potato virus Y (PVY<sup>NTN</sup>) of the resistant potato cultivar Sante, which harbors the Ry<sub>sto</sub> extreme resistance gene, did not induce ABA. Instead, jasmonic acid (JA) increased within the first few hours after PVY<sup>NTN</sup> infection (Flis et al., 2005; Kovac et al., 2009). Unaltered ABA content has also been reported for the resistant potato cultivar Rywal (carrying the *R*-gene Ny-1) following PVY infection, and for a resistant tomato cultivar (carrying the *R*-gene Tm-1) infected with TMV, although, in this latter case, the tomato cultivar resistant to TMV contained more ABA than a susceptible cultivar (Whennham et al., 1986; Baebler et al., 2014). Another study has shown that infecting resistant soybean (carrying the

*R*-gene Rsv3) with an avirulent strain (G5H) of soybean mosaic virus (SMV) resulted in higher ABA content during the first 24 h of infection. Interestingly, SA was not induced throughout the time course of the experiment, but was increased late in response to a virulent SMV strain (G7H) (Seo et al., 2014).

Although viroids represent an interesting class of infectious entities without encoded proteins, studies on defense responses to viroids are still preliminary and lack solid conclusions on the roles of ABA or other hormones. For example, in response to potato spindle tuber viroid (PSTVd) infection (RG1 severe strain), ABA-related genes have shown different patterns of expression in tomato cultivars. Some genes in the ABA biosynthesis pathway were upregulated, such as the subunit of farnesyl transferase and the phospholipase D  $\alpha$ -1, whereas few components of the guard cell ABA signaling pathway were downregulated (Owens et al., 2012). A similar study showed that no ABA or SA genes were induced following infection with the PSTVd RG1 strain, but only  $\beta$ -1,3-glucanase was induced at 25 days post-infection (Itaya et al., 2002). The difference between these two studies may be attributable to annotation of the tomato genome, which was not available at the time of the latter study. However, given the documented effect of ABA on callose accumulation, it can be speculated that ABA contributes to defense against viroids through callose. We will discuss the example of chrysanthemum stunt viroid (CSVd) spread in apical domains in the following section.

Since SA plays a major role in *R*-gene-mediated resistance, it is taken for granted that SA levels are elevated following viral infections (Baebler et al., 2014; de Ronde et al., 2014). However, there are some cases where JA or ABA are increased during early responses, such as of PVY<sup>NTN</sup> or SMV (Kovac et al., 2009; Baebler et al., 2014; Seo et al., 2014). In both examples, SA was induced at later stages of infection. This concurrent induction of ABA/JA then SA suggests that each hormone contributes differently to defense. It remains unanswered why hormone responses in incompatible interactions differ according to the infecting virus.

Abscisic acid deficiency has been reported to have an influential role in *R*-gene-mediated resistance against bacterial pathogens. For example, high temperature inhibits nuclear localization of the proteins SNC1 and RSP4, which is required for resistance against the bacterial pathogen *Pseudomonas syringae*. However, when the ABA biosynthesis pathway was impaired, nuclear localization of both proteins was enhanced regardless of temperature, leading to temperature-insensitive resistance against *P. syringae* (Mang et al., 2012). Since the effect of ABA was achieved through the biosynthesis pathway (by testing *aba1* and *aba2* mutants) rather than through ABA signaling (by testing *abi1-1* and *abi4-1* mutants), the authors suggested a role for ABA2 in *R*-gene-mediated resistance (Mang et al., 2012). Similar effects of ABA on *R*-genes that function against viruses are possible. Some *R*-genes have previously been shown to be temperature-sensitive, such as the *Rx*-gene against potato virus X (PVX) and the *N*-gene against TMV, but when plant culture temperatures were increased from 22 to 28°C the hypersensitive response disappeared in infected tobacco and tomato plants (Samuel, 1931; Whitham et al., 1996; Wang et al., 2009). A recent

study revealed that the temperature-sensitive *Wsm1* gene, which confers resistance to wheat streak mosaic virus (WSMV) and triticum mosaic virus (TriMV), and *Wsm2* that confers resistance to WSMV alone, block the systemic movement of both viruses in wheat at low temperature. Both viruses failed to enter the leaf sheaths of inoculated leaves at 18°C (but not at 24°C), thereby conferring resistance by impairing their long-distance movement (Tatineni et al., 2016). Whether or not ABA mediates these effects has yet to be investigated.

## ABA-DEPENDENT CALLOSE ACCUMULATION IS AN ANTIVIRAL MECHANISM

Plant viruses move from cell to cell via PD, with specific viral proteins (mostly movement proteins) modifying PD and increasing the size exclusion limit (which determines the size of the molecules traversing PD), thereby allowing the large viral movement complex to pass through (Fridborg et al., 2003; Lucas, 2006; Su et al., 2010; Heinlein, 2015). Trafficking through PD can be modulated by the controlled deposition of callose, a polysaccharide of the class  $\beta$ -1,3-glucan, at the necks of PD (Iglesias and Meins, 2000; Li et al., 2012b). Callose is a key component involved in cell fortification, and is found in different tissues at various developmental stages because it is required for growth and development. It is encoded by *callose synthase* (*CalS*) genes (or *glucan synthase-like* [*gsl*]), a gene family comprising 12 members in *Arabidopsis* that are involved in producing callose in different tissues/organelles (Verma and Hong, 2001; Dong et al., 2008; Ellinger and Voigt, 2014). Callose is also involved in plant response to biotic stress, with its deposition on the cell wall and at PD being important for restricting pathogen progression (Mauch-Mani and Mauch, 2005; Luna et al., 2011; Ellinger and Voigt, 2014). Among *CalS* genes, *CalS10* (or *GSL8*) has been identified as the primary regulator of callose deposition at PD (Guseman et al., 2010; Ellinger and Voigt, 2014; Han et al., 2014).

Plants control callose levels by the action of  $\beta$ -1,3-glucanases, which are hydrolytic enzymes that catalyze cleavage the 1,3- $\beta$ -D-glucosidic linkages into single  $\beta$ -1,3-glucan units (Doxey et al., 2007; De Strome and Geelen, 2014).  $\beta$ -1,3-glucanases are a diverse group of enzymes of different sizes, structure and localization (Doxey et al., 2007), and genes encoding these enzymes (e.g., PR2) are known to be induced during viral infections, which result in the removal of callose and thereby facilitates viral trafficking (Rezzonico et al., 1998; Kitajima and Sato, 1999; Oide et al., 2013).

In contrast, ABA has been shown to suppress expression of PR2, which allows more callose to accumulate at PD (Rezzonico et al., 1998) and thereby reduces viral intercellular movement and spread (Iglesias and Meins, 2000; Heinlein, 2015). The negative effect of ABA on  $\beta$ -1,3 glucanases suggests that ABA can increase callose accumulation in different tissues and organelles (PD, cell wall, phloem sieve plates). In fact, few studies listed below have shown the link between ABA induction, callose deposition and restriction of virus movement.

Below, we summarize the findings on the roles of callose in both compatible and incompatible plant-virus interactions:

## Roles of Callose in Compatible Interactions

Most of the cases reporting a role for ABA in plant defense against viruses involve compatible interactions. ABA pretreatment has been shown to reduce levels of different RNA viruses, such as tobacco necrosis virus (TNV) on *Phaseolus vulgaris* (Iriti and Faoro, 2008), TMV on *N. tabacum* (Whenham et al., 1986; Fraser and Whenham, 1989), and BaMV on *A. thaliana* (Alazem et al., 2014). These works postulated that enhanced callose deposition at PD could explain the ABA-dependent resistance, which is supported by the inability of TNV, for example, to spread in ABA-treated leaves (Iriti and Faoro, 2008).

In compatible interactions, the response of plants to virus or viroid infections is not strong enough to prevent spread of the viral agents to other tissues, which is evident from the levels of defense responses such as ABA, SA, callose and reactive oxygen species (ROS) (Kovac et al., 2009; Baehler et al., 2014; Seo et al., 2014; Lopez-Gresa et al., 2016). Considering that the biosynthesis pathway of ABA (like other hormones such as SA and JA) takes place in the chloroplast (Finkelstein, 2013), and that certain viruses and viroids interfere with several machineries in such plastids (Zhao et al., 2016), this might be the reason why some plants do not produce sufficient amounts of ABA or callose in response to infection in leaves. In contrast, callose deposition in meristematic tissues seems to be more efficient in preventing viroid spread. For instance, the response of two different *Argyranthemum* cultivars (Yellow Empire and Border Dark Red) to infection with chrysanthemum stunt viroid (CSVd) revealed that less callose was deposited at PD in the shoot apical meristem (SAM) of Yellow Empire compared to Border Dark Red, which resulted in the spread of CSVd to the uppermost cell layers in the apical dome and the youngest leaf primordia 1 and 2 of Yellow Empire (Zhang Z. et al., 2015). However, the SAM in the Border Dark Red cultivar presented more callose particles, which prevented CSVd from spreading beyond the lower part of the apical domain and elder leaf primordia (Zhang Z. et al., 2015). Which factor controls or induces callose deposition in SAM is unknown. Notably, both cultivars showed disease symptoms after infection with CSVd, which raises the question of whether callose deposition at PD occurs in other tissues (such as leaves) and whether this accumulation affects CSVd movement (Flores, 2016).

## Roles of Callose in Incompatible Interactions

Callose deposition has been documented in resistant soybean plants (carrying the *R*-resistance gene) in response to SMV. This response restricted SMV to the inoculated sites as no SMV RNA was detected beyond these sites (Li et al., 2012b). The same study also showed that susceptible soybean plants infected with SMV could not accumulate callose and, as a

result, SMV infection spread (Li et al., 2012b). A similar study showed that another soybean cultivar that possessed the *Rsv3* gene exhibited extreme resistance to SMV (Seo et al., 2014). This resistance was achieved by a subset of *PP2C*-encoding genes that comprise components of the ABA signaling pathway and that are induced by ABA. Recognition of SMV's cylindrical inclusion effector by the cultivar's *Rsv3* protein induced the ABA pathway and activated the *PP2Ca3* gene which, in turn, induced callose deposition and conferred extreme resistance against SMV (Seo et al., 2014). However, the mechanism linking *PP2C* proteins and *CalS* genes (or their protein products) or  $\beta$ -1,3-glucanases is unknown. Thus, induction of ABA in some incompatible plant–virus interactions suggests a role for ABA in innate immunity that needs to be experimentally validated (Whenham et al., 1986; Melotto et al., 2008; Kovac et al., 2009; Pacheco et al., 2012; Seo et al., 2014).

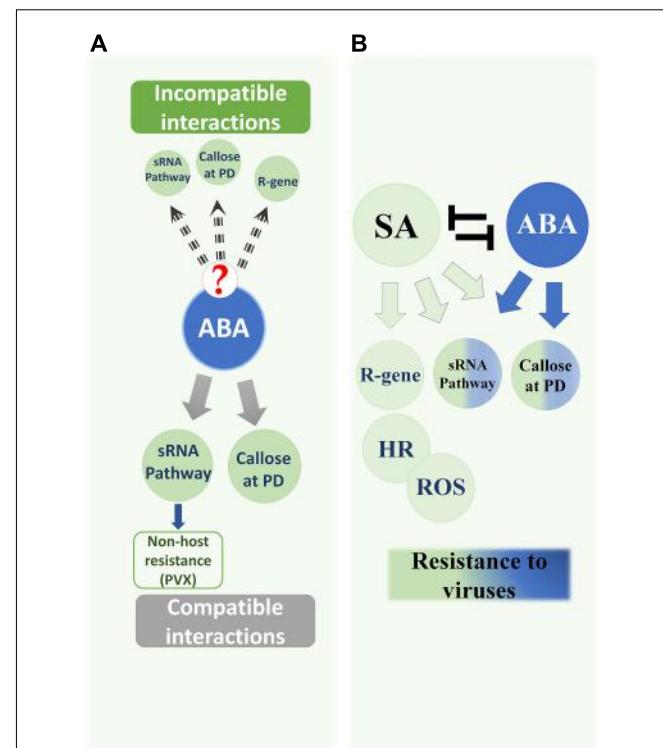
## Callose in the Early Antiviral Response: Is it Controlled by SA or ABA?

Early induction of ABA in some incompatible interactions supports the hypothesis that ABA plays a role during early immune responses against some viruses (Whenham et al., 1986; Melotto et al., 2008; Pacheco et al., 2012; Seo et al., 2014). However, it remains unclear whether or not callose deposition at that stage is completely ABA-dependent because no ABA mutants have been assessed to confirm the role of ABA-dependent callose deposition in incompatible interactions (Figure 1A).

In contrast, much more is known about how SA affects PD and callose. Several reports have shown that SA induces PD closure and impairs their permeability by increasing the amount of callose deposited at PD (Wang et al., 2013; Cui and Lee, 2016). This effect requires the action of plasmodesmata-located protein 5 (PDLP5), which is dependent on NPR-1 (Wang et al., 2013). PDLP5 controls the expression of *CalS1* and *CalS8* genes that are responsible for callose synthesis and deposition at PD in response to SA treatment (Cui and Lee, 2016). The major gene involved in callose deposition at PD, *CalS10*, functions independently of PDLP5 or SA, as evidenced by the normal plasmodesmal permeability induced by exogenous SA in the *cals10-1* mutant (Cui and Lee, 2016).

Despite the fact that both SA and ABA enhance callose deposition at PD (Figure 1B), the mechanism regulating this effect is quite different in each case. While the action of SA is mediated directly via specific genes (*PDLP5*, *CalS1*, and *CalS8*), ABA exerts a general indirect effect by transcriptionally decreasing  $\beta$ -1,3-glucanases that proteins may target all kinds of callose (Oide et al., 2013; Wang et al., 2013; Cui and Lee, 2016).

It is important to note that, in some cases, ABA does not lead to more callose deposition and, depending on growth conditions, its effect on callose deposition can even be reversed. For example, under conditions of low light intensity, high sucrose levels and the addition of



**FIGURE 1 |** Antiviral Roles of ABA and SA in plants. **(A)** ABA's documented roles against viruses in incompatible interactions: (i) Enhanced callose deposition at plasmodesmata (PD). (ii) Positive regulation of several AGO genes in the sRNA pathway, which reduces BaMV and PVX levels. ABA has an additional role in non-host resistance against PVX because ABA deficiency resulted in limited accumulation of AGO2 so that *Arabidopsis* became susceptible to PVX accumulation and systemic movement. The role of ABA in incompatible interactions has not been addressed. However, the effects of ABA on the callose and sRNA pathway, as well as the increased ABA content in some incompatible interactions, may suggest a role in such interactions. **(B)** The antagonistic pathways SA and ABA positively regulate common subsets of antiviral resistance mechanisms: callose deposition and sRNA (half-green half-blue circles). SA controls *R*-gene resistance, induces hypersensitive responses (HR), and the accumulation of reactive oxygen species (ROS) (green circles). SA contributes to the production of siRNAs and enhances callose deposition during early immune responses in incompatible interactions. In addition, exogenous application of SA increases plant tolerance to viruses in compatible interactions, which is supported by the increased susceptibility in lines with an impaired SA pathway.

Gamborg's vitamin to growth medium, applications of ABA have been shown to repress callose deposition (Luna et al., 2011).

## Suppression of Callose-Mediated Defense

Although ABA reduces the expression of  $\beta$ -1,3-glucanases, which are responsible for callose degradation, some viroids have evolved different ways to overcome the potential increase in callose deposition at PD. For example, PSTVd in tomato benefits from the activation of the small RNA (sRNA) pathway that produces sRNAs derived from the virulence modulating region of PSTVd. These viroid-derived sRNAs target the *CalS11-like*

and *CalS12-like* genes to interfere with callose synthase mRNA levels. However, their roles in callose accumulation at PD in tomato plants is unknown (Adkar-Purushothama et al., 2015a). In addition, some viruses recruit host factors that help degrade or remove callose from PD such as TAG4.4/SAG2.3, AtBG\_ppap (a beta-glucanase), ANK/TIP1-3, or others, so that callose does not hinder viral intercellular trafficking (Burch-Smith and Zambryski, 2016).

Several gaps remain in our knowledge of the roles of the antagonistic ABA and SA pathways in callose-mediated restriction of virus spread in incompatible interactions. Some studies addressed the roles of either hormone in incompatible interactions and callose deposition (**Figure 1B**). However, since both hormones appear to affect callose levels, a study that jointly tests the effects of both hormones on  $\beta$ -1,3-glucanase and *CalS* genes and proteins, and consequent callose accumulation at PD or cell walls, would greatly clarify how cells react early to infection and induce defense responses.

## ABA-DEPENDENT ANTIVIRAL DEFENSE THROUGH RNA SILENCING PATHWAYS

Because sRNAs are repressors of gene expression, their mechanism of action is referred as RNA silencing, gene silencing, or RNA interference (Vaucheret, 2006). RNA silencing occurs on two levels; transcriptional gene silencing, and RNA degradation [or post-transcriptional gene silencing (PTGS)], which correlates with the accumulation of short-interference small RNAs (siRNAs) (Vaucheret, 2006). siRNAs are loaded into the RNA-Induced Silencing Complex (RISC) and guide Argonaute (AGO) proteins (the key player in RISC) to cleave or inactivate RNAs derived from transposons, viral-, trans-, or endogenous- genes leading to their degradation (Vaucheret, 2006; Chapman and Carrington, 2007). In *Arabidopsis*, the backbone of the RNA silencing pathway consists of proteins from three families: (1) The Dicer-Like (DCL) family, which comprises four genes (*DCL1*, *DCL2*, *DCL3*, and *DCL4*). (2) The AGO family, which comprises of 10 functional members (From *AGO1* to *AGO10*, with a pseudo *AGO8*) (Takeda et al., 2008; Mallory and Vaucheret, 2010; Seo et al., 2013). (3) The RNA-directed RNA polymerase (RDR)s family, which comprises three functional genes; *RDR1*, *RDR2*, and *RDR6*. The antiviral RNA silencing pathway is PTGS-based, and several genes in the DCL, AGO, and RDR families appear to have redundancy in their function against invading viruses (Vaucheret, 2008; Garcia-Ruiz et al., 2010, 2015; Pelaez and Sanchez, 2013; Seo et al., 2013). While siRNAs, which are derived from viruses, transgenes or from a subset of endogenous genes, are *cis*-acting siRNAs and therefore their action is termed as autosilencing, micro-RNAs (miRNA) originate from distinct genes, different from the ones they regulate, with their action referred to as heterosilencing (Bartel, 2004; Vaucheret, 2006). Viruses have evolved viral suppressors for RNA silencing (VSR) that enable them to counteract the antiviral RNA silencing pathway (Li and Ding, 2006; Burgyan and Havelda, 2011). Generally, VSR are multifunctional and play vital roles in viruses' movement,

replication or pathogenesis (Cao et al., 2010; Csorba et al., 2015). For example, the movement protein "triple gene block protein 1" in several potexviruses has VSR function along with its role in virus movement (Senshu et al., 2009; Lim et al., 2010; Brosseau and Moffett, 2015). Viruses often encode one VSR that can interfere the RNA silencing pathway at different steps such as binding dsRNA, preventing siRNA translocation or RISC assembly, or interacting with AGO proteins and impairing their silencing function (Li and Ding, 2006; Jin and Zhu, 2010; Burgyan and Havelda, 2011; Kenesi et al., 2017).

Until very recently, ABA-dependent callose deposition at PD was the only documented link between ABA and resistance to viruses. However, a recently revealed connection between ABA and the RNA silencing pathway has added another role for ABA in resistance to viruses (Alazem and Lin, 2015; Alazem et al., 2017). ABA-dependent defense against BaMV and PVX in *Arabidopsis*, for example, is mainly achieved through the RNA silencing pathway, not through callose deposition at PD (Jaubert et al., 2011; Alazem et al., 2017).

## Role of ABA in Endogenous sRNA Pathways

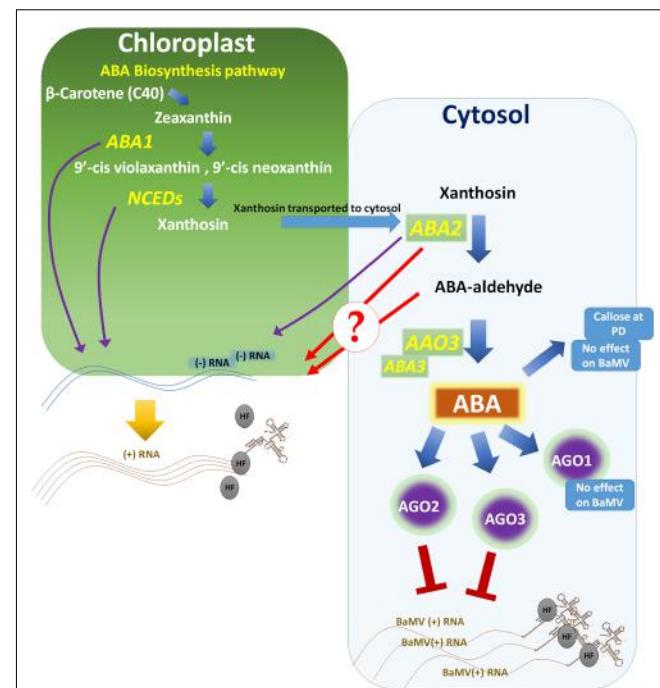
Expanding evidence has attributed a regulatory role for ABA in sRNA pathways, such as the siRNA and miRNA pathways. Previous works reported that ABA is required for stabilization of Cap binding proteins (CBP) 20 and 80 in a post-translational mechanism (Kim et al., 2008). These two proteins function in the formation of pre-miRNA transcripts and facilitate splicing during miRNA biogenesis. In addition, *cbp20* and *cbp80* mutants render plants hypersensitive to ABA. It is known that CBP20 is a negative regulator of ABA-dependent drought tolerance, and mutation of this gene renders plants tolerant to drought (Papp et al., 2004; Kim et al., 2008; Kuhn et al., 2008). Similarly, *CBP80* downregulation in potato reduced miR159 levels, thereby allowing accumulation of the miR159-target genes *MYB33* and *101* and consequently increasing drought tolerance (Pieczynski et al., 2013). In fact, mutants of several components of the miRNA pathway such as hyponastic leaves 1 (*HYL1*), HUA enhancer 1 (*HEN1*) or *DCL1* also exhibit hypersensitivity to ABA (Lu and Fedoroff, 2000; Zhang et al., 2008). Other mutants have shown ABA supersensitivity such as *dcl2*, *dcl3*, *dcl4* and their corresponding triple mutant. Expression of ABA-responsive genes such as *RD22* and *ABF3* was significantly increased in all *dcl* mutants. The mutants *dcl2*, *dcl3* and *dcl4*, but not *dcl1*, showed increased levels of *ABI3*, *ABI4*, and *ABI5* gene products (Zhang et al., 2008). Of note, *abi3-1* and *abi4-1* increased plant susceptibility to BaMV infection, but the genes regulated by these factors are still unknown (Alazem et al., 2014). Actually, several works have indicated that abiotic stresses such as drought, salinity, or cold stress (all of which are partially regulated by ABA) induce genes in the DCL and RDR families in tomato, maize and *Populus trichocarpa* (Qian et al., 2011; Bai et al., 2012; Zhao et al., 2015). The direct effect of ABA on the expression of DCLs or RDR is exemplified by the increased expression of *RDR1* in *A. thaliana* and of all *RDRs* in *O. sativa*, but only *RDR6*

was responsible for persistent ABA post-transcriptional control of gene silencing in *O. sativa* (Yang et al., 2008; Hunter et al., 2013).

## Antiviral Role of ABA through Regulation of AGOs

Argonaute proteins are integral players in all sRNA pathways in plants and animals, comprising a family of 10 members in *Arabidopsis* (Carbonell, 2017). By associating with different sRNAs, they regulate the expression of many genes and thereby control several aspects of growth, development and resistance to viruses (Vaucheret, 2008; Carbonell and Carrington, 2015; Zhang H. et al., 2015). All AGOs have been reported to reduce levels of different viruses, with variations in efficiency probably due to the effects of VSRs (Brosseau and Moffett, 2015; Carbonell and Carrington, 2015; Brosseau et al., 2016; Alazem et al., 2017). For example, when deleting the VSR of PVX ( $\Delta$ P25), all overexpressed AGOs downregulated PVX- $\Delta$ P25 in *N. benthamiana* (Brosseau and Moffett, 2015). miR168 levels, which maintains *AGO1* homeostasis, are regulated by ABA (Li et al., 2012a). Li et al. (2012a) found that *AGO1* RNA is not induced within 12 h of ABA treatment in *Arabidopsis* seedlings because of the effect of miR168, but another study found that extending the effect of ABA to 4 days induced not only *AGO1* but also *AGO2* and *AGO3* (Alazem et al., 2017). The latter study conducted experiments on ~30 day-old *Arabidopsis*, compared to the 7-day old seedlings used by Li et al. (2012a). These results were confirmed in ABA-deficient mutants (*aba2-1* and *aa03*), showing that *AGO1*, *AGO2*, and *AGO3* were expressed at very low levels (Alazem et al., 2017). In that study, BaMV infection also induced *AGO1*, 2 and 3 expression, but when *aba2-1*- and *aa03*-deficient mutants were infected with BaMV, *AGO1* and 2 but not 3 failed to accumulate to wild-type levels, indicating the expression of these AGOs is ABA-dependent. Furthermore, ABA was found to have negative effects on *AGO4* and *AGO10* expression, but differential effects on *AGO7* expression, since ABA treatment did not induce *AGO7* mRNA accumulation in wild-type plants but ABA-deficient mutants (*aba2-1* and *aa03*) showed significantly reduced expression of *AGO7* (Alazem et al., 2017). These findings imply that ABA generally affects several genes in the RNA silencing pathway, perhaps representing an important tool by which ABA tunes plant responses to different stimuli.

Although *AGO1* has antiviral activity against several viruses (Morel et al., 2002; Qu et al., 2008), BaMV levels were not reduced in the *4mAGO1* transgenic line in which *AGO1* was made resistant to the downregulatory effect of the *AGO1*-miR168 complex by four mismatches that prevent binding with miR168a. In the same context, the miR168a-2 mutant accumulates the *AGO1* protein, but BaMV levels were still unaffected in this mutant compared to wild-type plants (Vaucheret, 2009; Alazem et al., 2017). It could be that either *AGO1* has no clear effect against BaMV or that a VSR of BaMV (probably TGBp1) impairs the antiviral activity of *AGO1*. In contrast, the *ago1-27* mutant showed reduced BaMV levels compared to wild-type plants because of the increased expression of *AGO2*



**FIGURE 2 |** Abscisic acid (ABA) effects pathway on BaMV accumulation and plant antiviral resistance. A large part of ABA biosynthesis takes place in the chloroplast. Impairment of genes that function in the chloroplast, such as *ABA1* in *Nicotiana benthamiana* or *NCED3* in *Arabidopsis thaliana*, significantly reduces BaMV levels. The last two steps in ABA biosynthesis take place in the cytosol, where *ABA2* converts xanthosin into ABA-aldehyde and *AAO3* reduces ABA-aldehyde to produce ABA. *ABA2* mutants have markedly reduced levels of BaMV (-)RNA. Whether *ABA2*, ABA-aldehyde or other factors controlled by *ABA2* are required for BaMV to accumulate is unknown. In contrast, mutation of *AAO3* and downstream genes increases susceptibility to BaMV. ABA partially controls the expression of the AGO gene family and induces *AGO1*, 2 and 3, with *AGO2* and 3 but not *AGO1* acting against BaMV. In addition, ABA induction of callose is ineffective against BaMV because plants silenced in *CalS10* still show resistance after ABA treatment. HF, host factor.

and *AGO3* levels in this mutant. Surprisingly, BaMV levels were not affected in the ABA-treated *ago3-2* mutant and were not significantly reduced in the ABA-treated *ago2-1* mutant compared with corresponding mock-treated mutant (Alazem et al., 2017). These findings imply that ABA-dependent resistance to BaMV is mainly achieved through *AGO2* and *AGO3*, and that callose deposition at PD may not be the main resistance mechanism controlled by ABA, at least in some compatible interactions. In fact, restriction of viruses to the sites of infection during incompatible interactions can also be ascribed to the activity of the RNA silencing pathway, and further studies on this topic could reveal much about the involvement of ABA in incompatible interactions. In the same context, it was found that the RNA silencing pathway controls the non-host resistance of *A. thaliana* to PVX infection, mainly through *AGO2* (Jaubert et al., 2011). This finding was also confirmed for the *aba2-1* mutant, which produces very little *AGO2*, thereby allowing PVX to accumulate locally and move systemically

compared to the scenario in wild-type plants (Alazem et al., 2017).

Several studies have addressed the roles of the RNA silencing pathway in resistance to viroids since RNA or DNA replication intermediates trigger this pathway (Minoia et al., 2014; Adkar-Purushothama et al., 2015b; Carbonell and Daros, 2017). Since ABA regulates several genes in this pathway, ABA could also play a role in mediating resistance to viroids. For example, Minoia et al. (2014) found that *A. thaliana* AGO1, AGO2, AGO2, AGO3, AGO4, AGO5, and AGO9 were loaded with PSTVd-derived sRNA in infected *N. benthamiana* plants. Given the regulatory role of ABA in AGO1, 2, and 3, it is possible that ABA may participate in resistance to viroids through these AGOs.

## ABA AND RECESSIVE RESISTANCE

Recessive resistance is defined as the loss of susceptibility when an important host factor required for virus replication is impaired (Hashimoto et al., 2016). To date, most of the discovered recessive-resistance genes belong to the translation initiation factor (eIF) 4E and eIF4G groups (Hashimoto et al., 2016). However, other host factors are involved in BaMV accumulation and they localize to the cytosol and chloroplast (Figure 2). Further information on those factors is described in a recent review (Huang et al., 2017). Here, we briefly focus on the chloroplast-related genes since ABA and other hormones are biosynthesized in chloroplasts.

Chloroplast phosphoglycerate kinase (cPGK) interacts with the 3'-untranslated region of BaMV to direct BaMV RNA to the chloroplasts, and silencing or mislocalization of cPGK significantly reduces BaMV levels (Lin et al., 2007; Cheng et al., 2013). BaMV Minus-strand (-) RNA has been detected within chloroplasts, which suggests localization of BaMV replication intermediates there (Lin et al., 2007; Cheng et al., 2013). In accordance with these findings, the *Arabidopsis* genotype *Cvi-0* comprises a natural recessive resistance gene, *rwm1*, which encodes a mutated cPGK protein and confers resistance to two potyviruses (watermelon mosaic virus and plum pox virus) but not to the potexvirus PVX or the cucumovirus CMV (Lin et al., 2007; Oubrahim et al., 2014; Poque et al., 2015). Furthermore, the ABA biosynthesis gene *ABA2* and the upstream gene *NCED3* are important for BaMV (-)RNA accumulation (Alazem et al., 2014). Because of the feedback loop in the ABA biosynthesis pathway, the *nced3* mutant exhibited low levels of ABA2, accounting for the low level of BaMV in that mutant. Hence, ABA2 is required for a step preceding BaMV translation, and a similar role was also suggested for the accumulation of CMV in *A. thaliana* (Alazem et al., 2014).

In the same context, in the ABA biosynthesis pathway, ABA1 and NCED3 are localized in the chloroplasts, whereas ABA2 and AAO3 (the *aao3* mutant is highly susceptibility to BaMV

unlike the *aba2-1* mutant; Alazem et al., 2014) are localized in the cytosol. Hence, the ABA biosynthesis pathway in the chloroplasts may be required for BaMV accumulation (Figure 2). It is still not known whether this recessive resistance is the result of ABA2 substrate or other factors controlled by ABA2. The different localization of cPGK and ABA2 (Cheng et al., 2002) and the different nature of the substrates handled by them may suggest different roles.

## CONCLUDING REMARKS

The increased expression of several genes of the *AGO*, *RDR*, and *DCL* families in response to ABA, as well as the observation that several of these genes are important players in the antiviral RNA silencing pathway, strengthens the notion that the antiviral role of ABA is partially achieved through the RNA silencing pathway. The additional effect of ABA-dependent callose deposition at PD thus endows ABA with a dual function in restricting virus spread (Figure 1A). Both mechanisms have been assessed only for BaMV (Figure 2), and the findings have shown that callose deposition is not the only defense mechanism mediated by ABA. Further studies with other viruses and viroids will reveal how efficient these mechanisms are in different pathosystems.

The antagonism between SA and ABA is well-documented, whereby downstream genes of either pathway are suppressed if the other hormone is applied or induced (Yasuda et al., 2008; Zabala et al., 2009; Moeder et al., 2010). It is known that viruses disrupt hormonal balance in compatible interactions, leading to simultaneous induction of some antagonistic pathways such as ABA and SA in the case of BaMV and CMV (Alazem et al., 2014). However, because of the positive effects that both hormones have on the same subset of defense responses (Figure 1B), it is not clear whether these two antagonistic pathways actually act antagonistically during viral infections. Antagonism is evident in some incompatible interactions in which the induction of these pathways is strong, sequential and not concurrent, implying that each hormone takes a role in triggering several redundant antiviral mechanisms (Alazem and Lin, 2015), but experimental evidence is lacking.

## AUTHOR CONTRIBUTIONS

MA and N-SL wrote, revised, and approved this manuscript.

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# Transmission of *Bamboo mosaic virus* in Bamboos Mediated by Insects in the Order Diptera

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*Bamboo mosaic virus* (BaMV), a member of the genus *Potexvirus*, is the major threat to bamboo cultivation. Similar to most potexviruses, the transmission of BaMV by insect vectors has not been documented previously. However, field observations of BaMV disease incidences suggested that insect vectors might be involved. In this study, we aimed to investigate the possibility of insect-mediated transmission of BaMV among bamboo clumps, in order to provide further insights into the infection cycles of BaMV for the development of effective disease management measures. From the major insects collected from infected bamboo plantations, BaMV genomic RNAs were detected inside the bodies of two dipteran insects, *Gastrozona fasciventris* and *Atherigona orientalis*, but not in thrips (*Scirtothrips dorsalis*). Artificial feeding assays using green fluorescent protein-tagged BaMV virions revealed that BaMV could enter the digestive systems and survive in the regurgitant and excretion of the dipterans. BaMV RNA could be retained in the dipterans for up to 4 weeks. Insect-mediated transmission assays indicated that both dipterans could transmit BaMV to bamboo seedlings through artificially created wounds with low infection efficiency (14 – 41%), suggesting that the dipterans may mediate the transmission in a mechanical-like manner. These results demonstrated that dipterans with sponge-like mouthparts may also serve as vectors for at least one potexvirus, BaMV, among bamboo plants. The finding suggested that dipteran insect control should be integrated into the disease management measures against BaMV infections.

**Keywords:** insect transmission, Diptera, *Gastrozona fasciventris*, *Atherigona orientalis*, *Potexvirus*, *Bamboo mosaic virus*, bamboo

## INTRODUCTION

Bamboos are economically important crops for their broad applications in both agriculture and industry. However, the cultivation of bamboo crops is under the threat of a major pathogen, *Bamboo mosaic virus* (BaMV), a member of the genus *Potexvirus* in the family *Flexiviridae* (Lin et al., 1992). BaMV has been reported to infect different bamboos in Brazil, Taiwan, California and Florida (USA), Australia, Hawaii, and Mainland China (Lin et al., 1977, 1979, 1993, 1995, 2014; Lin and Chen, 1991; Elliot and Zettler, 1996; Dodman and Thomas, 1999; Nelson and Borth, 2011). At least 10 commercially cultivated species of bamboos are susceptible to the infections of BaMV (Lin et al., 1993). Typical symptoms of infected bamboos include mosaic or chlorotic streaks in between the veins of the leaves, necrotic tissues (brown to black spots or streaks) in the shoots and culms,

aborted shoots, reduced vigor, and even death of the bamboo clumps (Lin et al., 1993; Hsu and Lin, 2004; Nelson and Borth, 2011). The necrotic tissues in the shoots greatly reduce the yield, quality, and value of the bamboo products, and are usually referred to by farmers as bamboo shoot “nails” due to the resemblance in appearance and texture. With a disease prevalence of about 70–80% in bamboo plantations in Taiwan (Lin et al., 1993), BaMV is recognized as one of the major limiting factors for bamboo cultivation.

Being a potexvirus, BaMV has the typical flexible filamentous virion structure (Dimaio et al., 2015), harboring a positive-sense, single-stranded RNA genome of about 6.4 kb in length, with a 5'-cap and a 3' poly(A) tail (Lin et al., 1992; Chen et al., 2005). BaMV is believed to spread through vegetative propagation of bamboos using seedlings produced from BaMV-infected bamboo mother stocks, or mechanical transmission by BaMV-contaminated tools used in harvesting or pruning (Elliot and Zettler, 1996; Hsu and Lin, 2004; Nelson and Borth, 2011). However, the possible involvement of other transmission routes, such as via insect vectors, has not been ruled out.

It has been considered that most of the potexviruses are not transmitted by insect vectors (Koenig and Lesemann, 1978), with only few exceptions in earlier archives (Schmutterer, 1961; Goth, 1962; Kassanis and Govier, 1971), including a distinct potexvirus, *Strawberry mild yellow edge associated virus* (SMYEv) that has been reported to be transmitted by aphids in a persistent mode (Jelkmann et al., 1990), although the involvement of a luteovirus (Yoshikawa et al., 1984; Martin and Converse, 1985; Spiegel et al., 1986) as the helper could not be ruled out (Jelkmann et al., 1990). Similar to most potexviruses, insect-mediated transmission of BaMV has not been reported previously. Thus, the control and prevention of insect pests are not included in the current recommendations for BaMV disease management (e.g., Nelson and Borth, 2011). However, field observations suggested that insect vectors may be involved in BaMV transmission among bamboos. Firstly, the BaMV-infected bamboo clumps in bamboo plantations are not usually distributed closely together. Rather, the patterns of the disease incidence are often sporadic, discontinuous, and not correlated to the route of harvesting or pruning. If BaMV is only transmitted by contaminated tools, one would expect to see disease incidences connected to the sources of contamination or along the paths of maintenance work. Secondly, the cut surfaces of the rootstocks of harvested bamboo shoots (**Figure 1A**) and the pruned culms attract large amounts of insects, mostly of the order *Diptera*, within minutes. The dipterans stay probing and feeding on the cut surfaces till the surfaces dry out if not disturbed, and fly on to the newly generated wounds of the bamboo clumps, possibly far away from the original clump as the harvesting or pruning processes proceed while the insects are feeding. If the dipterans are actually involved in BaMV transmission among bamboos, the lack of measures against insect pests may constitute a loophole in the current integrated disease management of BaMV.

In this study, we aimed to explore the possibility that insects may mediate the transmission of BaMV, a potexvirus, among bamboos. Several lines of evidence were provided in support

of the notion that at least two dipteran insects may serve as vectors for transmission of BaMV among bamboos. The results revealed the potential threat of the dipteran insects with sponge-like mouthparts as vectors for at least one plant virus, and suggested that the control of these insects should be integrated into the current systems for disease management against BaMV infections.

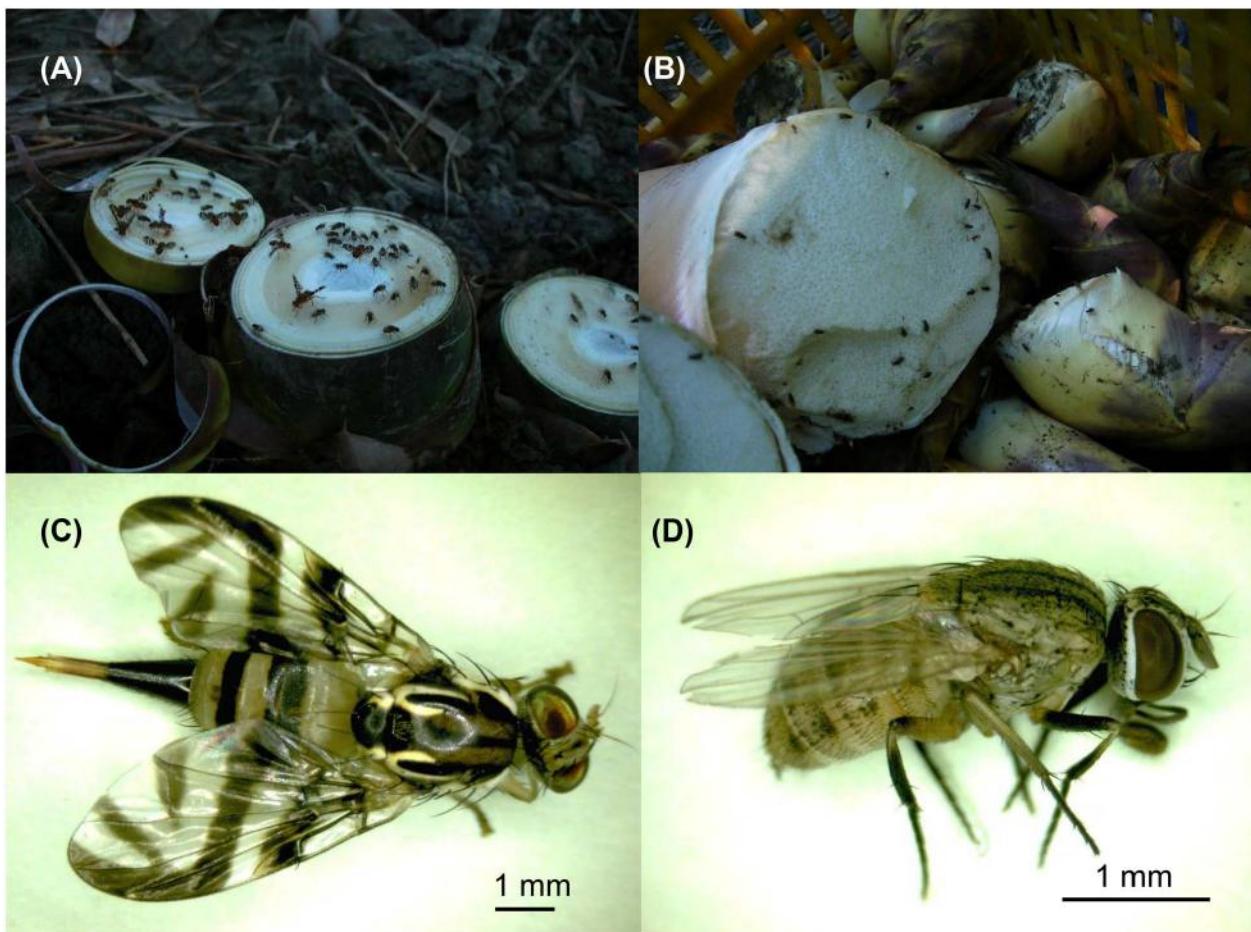
## MATERIALS AND METHODS

### Extraction of Total Nucleic Acids from Bamboo Tissues and Insects

To prevent the contamination of non-specific nucleic acids on the outer surfaces of the insects, RNase Away (Sigma-Aldrich, Shanghai, China) treatment was included as the first step of sample preparation. To determine the efficiency of RNase Away in removing the contaminating nucleic acids, BaMV-free *Gastrozona fasciventris* were anesthetized with acetone vapor and placed into 1.5-ml Eppendorf tubes containing 20  $\mu$ l of BaMV (0.1 mg/ml in 0.5 M sodium borate buffer, pH 8.0) individually. The tubes were then incubated on ice for 5 min to coat BaMV onto the surfaces of the insects and the tubes. Following the removal of BaMV solution by manual pipetting, RNase Away (200  $\mu$ l) was added to each tube. The tubes were then incubated at room temperature with gentle shaking for 1, 2, or 3 min, followed by thorough washing with 1 ml of de-ionized H<sub>2</sub>O for three times (3 min each). Then the samples were subjected to RNA extraction and analyses as described below. It was thus determined that the bamboo tissue and insect samples should be treated with RNase Away for 3 min to remove the contaminating nucleic acids on the surfaces before extraction of total nucleic acids (see Results). Total nucleic acids were extracted from bamboo or insect tissues according to the methods described previously (Haible et al., 2006), with minor modifications. Briefly, the RNase Away-treated bamboo (0.1 g leaf or shoot tissue) or insect (whole body) samples were ground to powders in liquid nitrogen and the total nucleic acids were extracted by the addition of 0.2 ml of extraction buffer (100 mM Tris-HCl pH 7.5; 1 mM EDTA; 100 mM NaCl; 100 mM DTT, 0.6% SDS) and equal volume of saturated phenol. After centrifugation at 12000  $\times$  g for 10 min, the supernatants were transferred to new tubes and the nucleic acids precipitated by the addition of 2.5 volume of 95% ethanol. The final nucleic acid pellets were re-suspended in 20  $\mu$ l of de-ionized H<sub>2</sub>O for further analyses.

### Molecular Identification of Dipteran Insects and Detection of BaMV RNA by Reverse-Transcription-Polymerase Chain Reaction (RT-PCR)

To identify the dipteran insects collected from bamboo plantations, the chromosomal *cytochrome oxidase I* (COI), 28S rDNA and, mitochondrial 16S rDNA gene segments were amplified by PCR using gene-specific primer pairs (**Table 1**), cloned, and sequenced following standard protocols described in the Barcode of Life Database (Ratnasingham and Hebert, 2013;



**FIGURE 1 | The main insect species collected in bamboo plantations in southern Taiwan.** (A) After the harvesting of the bamboo shoots, the cut surfaces exposed on the ground attracted many dipteran insects within minutes. (B) The surface of the harvested bamboo shoots also attracts some dipteran insects. The most abundant dipteran insects were identified as *Gastrozona fasciventris* (C) and *Atherigona orientalis* (D), respectively.

Smit et al., 2013). For the detection of BaMV genomic RNA, the aforementioned total nucleic acids (2.5  $\mu$ l) were used as the template in a mixture (10  $\mu$ l) containing 50 pmole of oligo dT primer. The template-primer mixtures were heated to 65°C for 2 min, snap-chilled on ice for 1 min, then transferred to a RT reaction containing 10 mM DTT, 0.25 mM dNTPs, and 5 units of SuperScript III reverse transcriptase (Thermo Fisher Scientific, Waltham, MA, USA). The RT reaction mixture was incubated at 37°C for 60 min, followed by inactivation at 85°C for 5 min. The cDNA products were used as the template in the polymerase chain reaction (PCR) for the detection of BaMV in bamboo tissue and dipteran insect samples using BaMV-specific primer pairs (**Table 1**). The PCR mixture (50  $\mu$ l) contained 5  $\mu$ l of 10 $\times$  rTaq buffer, 5  $\mu$ l of dNTP mixture (2.5 mM each), 0.25  $\mu$ M of specific primer pair (e.g., B-5981R plus B-6366), 1.0 unit of rTaq DNA polymerase (Toyobo Life Science, Osaka, Japan) and 1–2  $\mu$ l of the aforementioned total nucleic acids or cDNA products as the template. PCR was performed as follows: reaction mixtures were heated to 94°C for 4 min for initial denaturation, followed by 35 cycles of denaturing at 94°C for 40 s, annealing at 47°C for 45 s,

and extension at 72°C for 45 s, with a final extension at 72°C for 20 min. The PCR products were then analyzed by electrophoresis through a 1% agarose gel, stained with ethidium bromide (EtBr), and examined by UV illumination.

### Purification of Green Fluorescent Protein (GFP)-Tagged BaMV Virions

To track the ingestion of BaMV virions, a mutant BaMV construct that generates GFP-tagged BaMV virions in plants, designated pCB-GFP2a-CP, was constructed based on a mutant infectious clone of BaMV, pBS-d35CP (Yang et al., 2007). The coding sequences of FMDV 2A cotranslational dissociation peptide (LLNF DLLKLAGDVESNPGP) (Ryan et al., 1991) was amplified by PCR with primers FMDV2aF and FMDV2aR (**Table 1**), and inserted to the 5'-end of truncated CP ORF on pBS-d35CP following restriction digestion with *Bam*H1 and *Psp*OMI. The GFP coding region (Sheen et al., 1995) was amplified with primers GFP F and GFP R (**Table 1**), digested with *Bam*H1 and then inserted to the 5'-terminus of the FMDV 2A coding region to give pCB-GFP2a-CP. Due to the presence of

**TABLE 1 |** Primers used in this study.

| Name    | Sequence (5' to 3')   | Target gene           | Reference           |
|---------|---|-----------------------|---------------------|
| FMDV2aF | GGGATCCCGGGCCCGCGCT<br>GTTGAATTGACTTCTTA<br>AGCTTGCGGG              | FMDV 2A peptide       | Ryan et al., 1991   |
| FMDV2aR | CCT <u>GGGCCGGGTCCGGG</u><br>GTTGGACTCGACGTCCTC<br>CGCAAGCTTAAGAAGG | FMDV 2A peptide       | Ryan et al., 1991   |
| GFP F   | AAGGATCCATGGTGAGCAAGGG  | nts 1-14 of GFP       | This study          |
| GFP R   | TGGGATCCTTACTTGTACAGCTCG  | nts 702-717 of GFP    | This study          |
| 1R      | GAAAACCACTCCAAACG   | nts 1-17 of BaMV      | This study          |
| 331     | GGAGATATGAGGCCGTCCG   | nts 313-331 of BaMV   | This study          |
| 2934R   | ACTGCATCCAAACCGAAAAC  | nts 2934-2953 of BaMV | This study          |
| 3527    | TCTTGAGACTGGTCATACG   | nts 3509-3527 of BaMV | This study          |
| 5981R   | CACAATATAATGGTGTGCG   | nts 5981-6000 of BaMV | This study          |
| 6366    | TGGAAAAAACTGTAGAACCAAAAGG   | nts 6341-6366 of BaMV | This study          |
| 16S-F   | TAGTTTTTTAGAAATAATTAAATTAA  | 16S rDNA              | Smith et al., 2003  |
| 16S-R   | GCCTTCAATTAAAAGACTAA  | 16S rDNA              | Smith et al., 2003  |
| 28SS    | GACCCGTCTTGAAMCAMGGA  | 28S rDNA              | Asokan et al., 2013 |
| 28SA    | TCGGARGGAACCAGCTACTA  | 28S rDNA              | Asokan et al., 2013 |
| TEPCOIF | TAAACTTCAGCCATTAAATC  | COI                   | Smit et al., 2013   |
| TEPCOIR | TTTCCTGATTCTTGCTAA  | COI                   | Smit et al., 2013   |

FMDV 2A cotranslational dissociation peptide, the inoculation of pCB-GFP2a-CP in plants would generate both GFP-fused and unfused BaMV CP to assemble into GFP-tagged BaMV virions, together with free GFP-2a fusion proteins. The incorporation of FMDV 2A peptide would increase the assembly efficiency and stability of the GFP-tagged BaMV virions, compared to the construct without FMDV 2A peptide (Hsu et al., unpublished). Leaves of *Chenopodium quinoa* mechanically inoculated with purified plasmid pCB-GFP2a-CP DNA (1 µg/µl in de-ionized water, 10 µl/leaf, 5 leaves/plant) or wild type BaMV virions (0.1 µg/µl in de-ionized water, 10 µl/leaf, 5 leaves/plant) were harvested at 10 dpi. The leaves were ground in de-ionized water (1:10 w/v) to prepare the inoculum used for mass-inoculation of *C. quinoa* plants (50–100 plants/batch). At 10 dpi, the leaves were harvested, and the GFP-tagged or wild type BaMV virions were subsequently purified from the leaves as described by Lin and Chen (1991). The yield was determined spectrophotometrically by absorbance at 280 nm (Lin and Chen, 1991). Purified BaMV virions were dissolved in BE buffer (50 mM Borate, pH 8.0, 1 mM EDTA), then stored at –20°C until used. It should be noted that the purification procedure include an ultracentrifugation through a 5-ml 20% sucrose cushion, which precludes most of the unassembled viral proteins in the pellets (Muthamilselvan et al., 2016). However, the possibility of the presence of some unassembled BaMV CP, GFP-tagged BaMV CP, and GFP-2a fusion proteins in the virion preparations could not be ruled out.

## Dipteran Insect-Mediated Transmission

BaMV-free green bamboo (*Bambusa oldhamii*) seedlings originating from meristem tip-cultured bamboos (Hsu et al., 2000) were kindly provided by Dr. Choun-Sea Lin (Agricultural Biotechnology Research Center, Academia Sinica, Taiwan).

Alternatively, BaMV-free green bamboo seedlings propagated by air-layering on BaMV-free bamboo plants were obtained from a bamboo seedling plantation of Mr. Kuo-Chen Chang, which is regularly indexed for BaMV infection. The air-layered bamboo seedlings were used as the hosts for BaMV-transmission assays due to their ability to produce more bamboo shoots. To facilitate the feeding of the flies, the newly emerged bamboo shoots were cross-sectioned at the crown region to mimic the harvesting process. The dipterans were co-incubated with restrained the bamboo seedlings in 200-mesh insect domes for infection assays. For inoculation, 4–7 dipterans fed with liquid medium (10% sucrose, 2% yeast extract) supplemented with purified BaMV virions (0.1 mg/ml, mimicking the concentration of BaMV in bamboos) were released into the insect domes containing bamboo seedlings with wounds. After incubation for 24 h, the dipterans were removed manually, and the bamboo seedlings were maintained in the greenhouse or insect domes for at least 60 days until assayed. To confirm the BaMV-free condition of the test plants, the un-inoculated siblings of the tested bamboo seedlings from the same mother-stocks were assayed for the presence of BaMV following the inoculation assays by northern and western blot analyses.

## Northern, and Western Blot Analyses

To verify the infection and replication of BaMV in bamboo plants, northern and western blot analyses were performed as described by Huang et al. (2012) and Hung et al. (2014), respectively. For northern blot hybridization, <sup>32</sup>P-labeled probes specific for the 3' untranslated regions of BaMV RNAs (Hsu et al., 2000) were used to detect the presence of genomic and two major subgenomic RNAs, which are only transcribed following the successful replication of BaMV genomic RNAs. For western

**TABLE 2 | Detection of BaMV in two major dipterans found in different bamboo plantations in Taiwan.**

| Location                | <i>Atherigona orientalis</i> | <i>Gastrozona fasciventris</i> |
|-------------------------|------------------------------|--------------------------------|
| Baihe #1, Tainan City   | 25/26 <sup>a</sup>           | 18/24                          |
| Baihe #2, Tainan City   | 17/20                        | 5/9                            |
| Tanzi #1, Taichung City | 1/7                          | 6/14                           |
| Tanzi #2, Taichung City | 3/8                          | 5/7                            |
| Wugu, New Taipei City   | 10/11                        | 4/4                            |

<sup>a</sup>Number of BaMV-positive insects/number of tested insects.

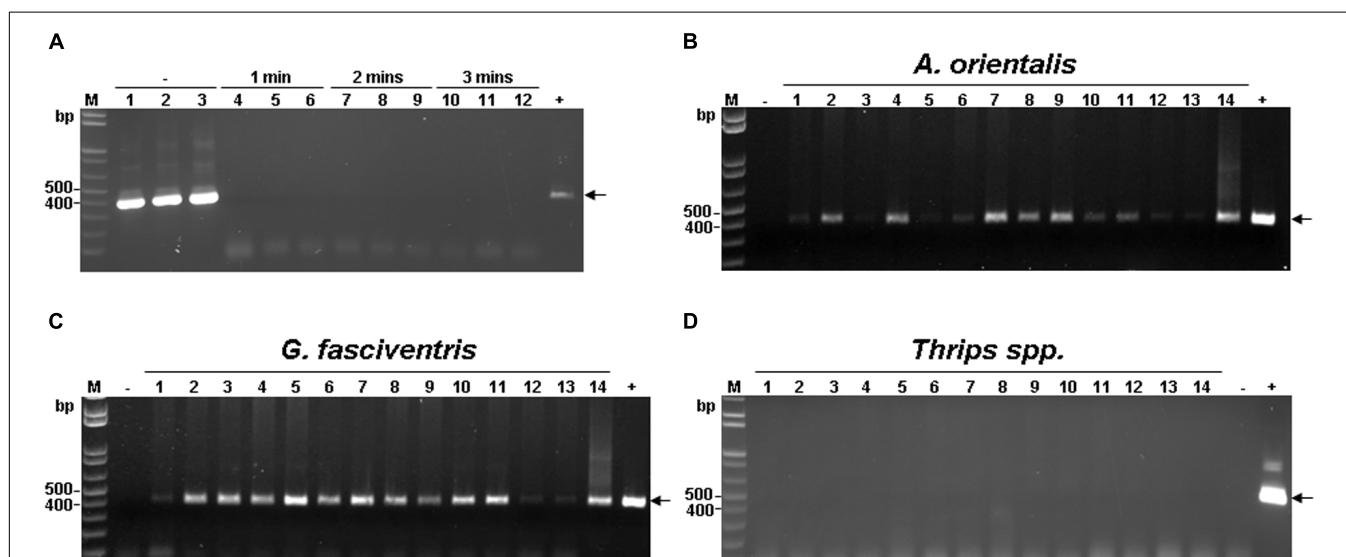
blot analysis, specific antibody (Hung et al., 2014) against the triple gene block protein 1 (TGBp1) of BaMV, which is translated from subgenomic RNA 1 and not present in the inoculum, was used to demonstrate successful infection and gene expression of BaMV in inoculated bamboos.

## RESULTS

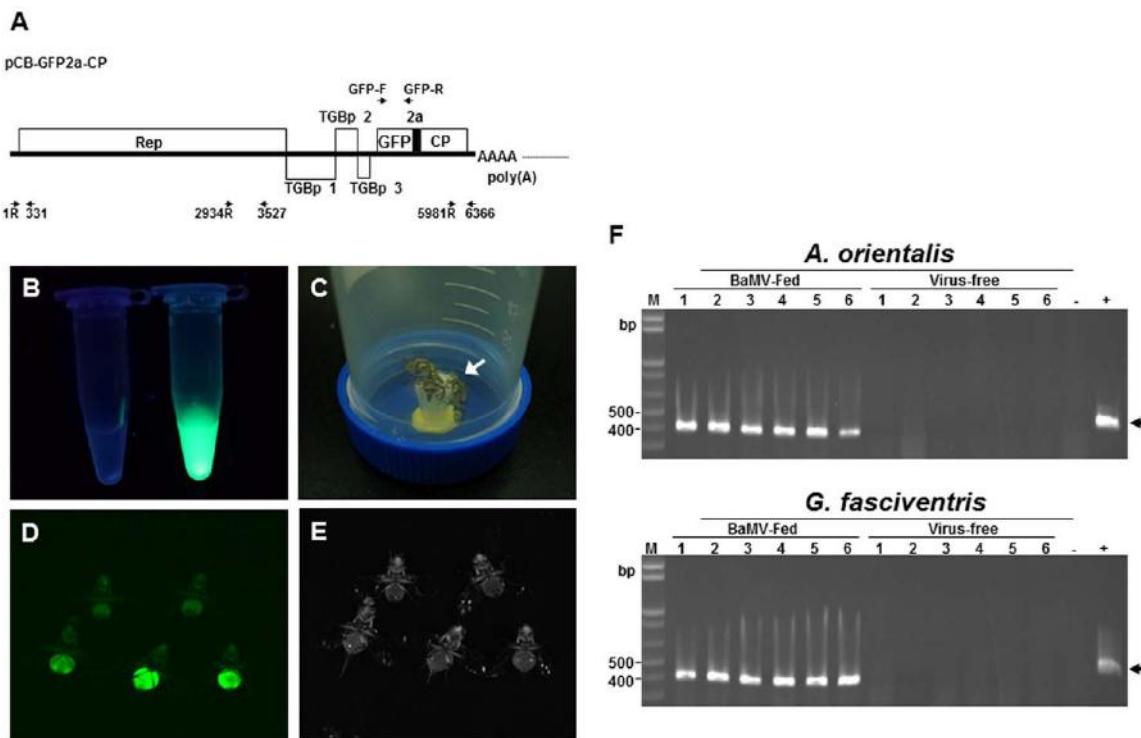
### BaMV Could be Detected in Two Major Dipteran Insect Species Collected from BaMV-Infected Bamboo Plantations

The harvesting, pruning, and other regular maintenance of bamboo crops create cut surfaces or wounds that attract many insects within minutes, mostly in the order *Diptera* (Figures 1A,B). Two major insect species were identified to be *Gastrozona fasciventris* (Figure 1C) and *Atherigona orientalis* (Figure 1D), based on the morphology, wing

markings (Permkam, 2005), and nucleotide sequences of the chromosomal COI, 28S rDNA and, mitochondrial 16S rDNA gene segments (Smith et al., 2003; Ratnasingham and Hebert, 2013; Smit et al., 2013). Other minor species included *Taenioptera vittigera*, *Stylocladius appendiculatus*, and *Drosophila melanogaster*, identified based on the morphologies. To test whether BaMV may have insect vectors, we collected insects from five bamboo plantations (Table 2) located in the major bamboo production areas in southern (two plantations in Baihe, Tainan City), central (two plantations in Tanzi, Taichung City), and northern Taiwan (one plantation in Wugu, New Taipei City). The BaMV disease incidences in these plantations ranged from 40 to 70%. RT-PCR was used to detect the presence of BaMV in the main insect species collected. RNase Away was used to avoid the contamination of BaMV on the outer surfaces. To determine the treatment conditions, a pre-test was performed using BaMV-coated *G. fasciventris*. As shown in Figure 2A, the treatment of RNase Away for 1 min was enough to remove the contamination of BaMV. For the following experiments, the insect samples were treated with RNase-Away for 3 min to ensure the removal of the possible contaminations of RNases and RNAs, and thoroughly rinsed with RNase-free de-ionized water before being subjected to total nucleic acid extraction. RT was performed using oligo-dT as the sole primer. The cDNA products were then subjected to PCR with primer pair specific to BaMV 3'-terminus (B-5981R and B-6366). The results revealed that BaMV could be detected in the dipteran insect samples, *A. orientalis* and *G. fasciventris*, but not in the thrips (*Scirtothrips dorsalis*) collected from the backside of the leaves of BaMV-infected bamboos (Figure 2). The results of the BaMV



**FIGURE 2 | Detection of BaMV RNA in insects collected from BaMV-infected bamboo plantation. (A)** Determination of RNase-Away treatment time. See Section “Materials and Methods” for details. Briefly, BaMV-coated *G. fasciventris* individuals were treated with RNase-Away for 1–3 min as shown on top of the lanes, or untreated (as indicated by the “−” sign), rinsed thoroughly and tested for presence of BaMV by RT-PCR by primers B-5981R and B-6366 (Table 1). The expected position of BaMV-specific products is indicated by the arrow. **(B–D)** Detection of BaMV RNA in insect samples by RT-PCR using the same condition as in **(A)**. Lane M, 1 kb ladder size marker; lanes 1 through 11, *A. orientalis* **(B)**, *G. fasciventris* **(C)** or *Thrips spp.* **(D)** samples; lanes 12, 13, surface and inner tissues of bamboo shoots, respectively; lane 14 bamboo leaf sample collected from BaMV-infected plantation. The plasmid pCB (1 ng, Yang et al., 2007) and total nucleic acids from BaMV-free bamboo samples were used as positive and negative controls (lanes + and −), respectively.



**FIGURE 3 |** Tracking the ingestion of BaMV in *G. fasciventris*. **(A)** Schematic representation of the plasmid pCB-GFP2a-CP. The coding sequences of FMDV 2A co-translational dissociation peptide (short black box) and GFP inserted at N-terminus of BaMV CP genes. The genome of BaMV is represented by the thick black line. ORFs were shown as boxes with identities indicated. The relative positions and directions of the primers used in **Figure 5** are also shown. **(B)** Purified GFP-tagged BaMV particles (1 mg/ml, tube on the right) under UV illumination (360–390 nm). Equal volume of the wild type BaMV was shown on the left. **(C)** Virus-feeding treatment. Groups of two to seven *G. fasciventris* flies were fed with media containing GFP-tagged BaMV through the cotton matrix (indicated by the arrow) affixed to the cap of the tube. **(D,E)** Examination of the ingestion of GFP-tagged BaMV in *G. fasciventris*. The flies were fed with liquid medium supplemented with wild type BaMV (two flies in the upper row) or GFP-tagged BaMV (three flies in the lower row) for 24 h, anesthetized by acetone vapor, and examined by illumination of blue light (485 nm, **D**) or white light (**E**). Note that the flies were only slightly anesthetized by acetone for imaging to retain their ability for feeding and flying in the virus-transmission assay, which led to the movement of some of the flies in the matching photos in **(D,E)**. The flies were turned upside down to show the ventral views. **(F)** Detection of BaMV RNA in *A. orientalis* and *G. fasciventris* flies fed with BaMV-containing medium. To test the ability of dipteran insects in acquiring BaMV through feeding, *A. orientalis* and *G. fasciventris* flies were fed with BaMV-containing medium (10% sucrose, 2% yeast extract, and 0.01% BaMV) or virus-free medium for 24 h, as indicated on top of the lanes. The presence of BaMV in insect bodies was assayed by RT-PCR as described above.

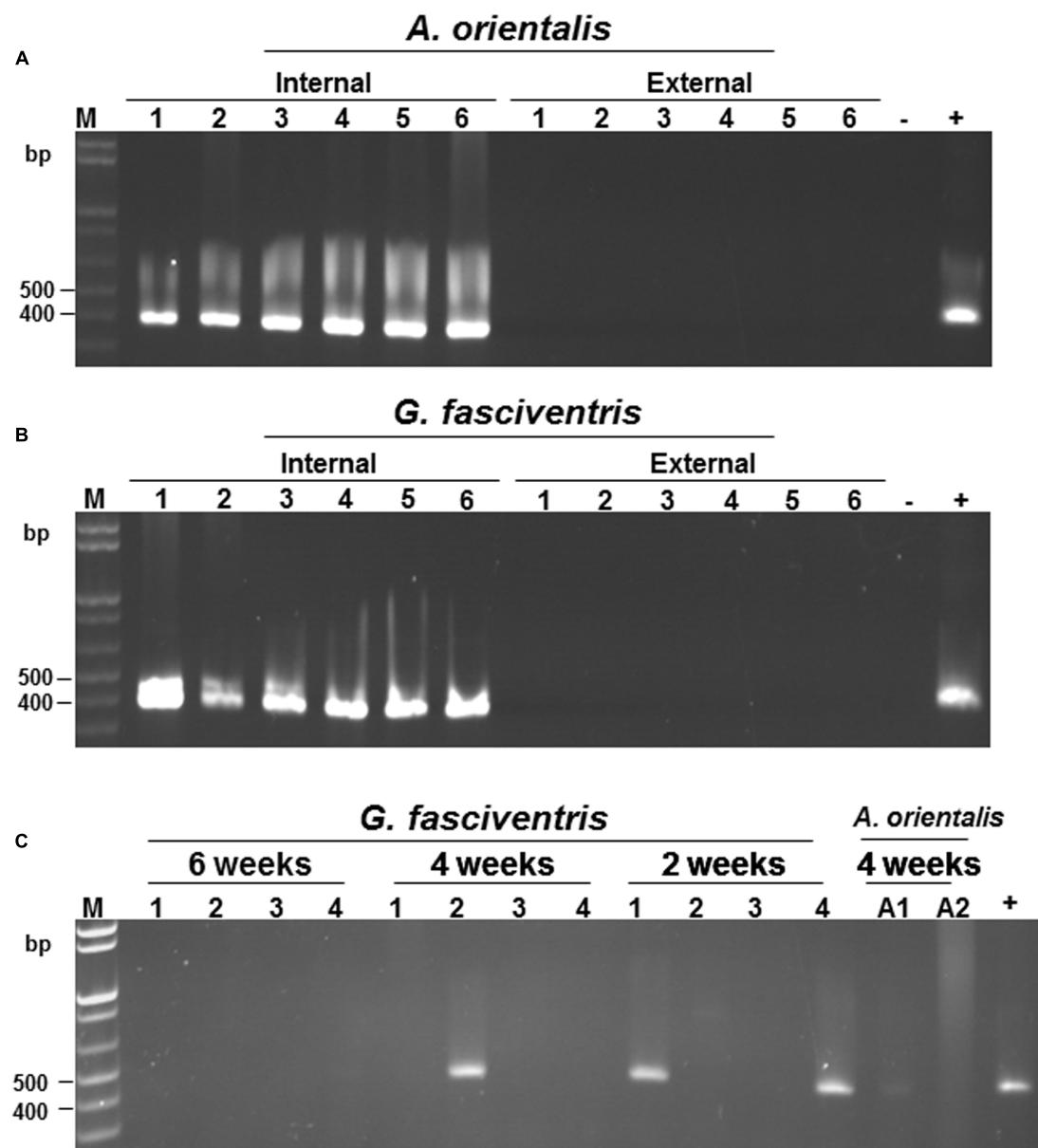
detection in *A. orientalis* and *G. fasciventris* collected from the five bamboo plantations were summarized in **Table 2**. BaMV detection was not performed on *T. vittigera*, *S. appendiculatus*, and *D. melanogaster*, since these minor insects were not always trapped in all bamboo plantations surveyed, and thus not included in further analyses.

To further explore the possibility that the dipteran insects may mediate the transmission of BaMV, we established BaMV-free colonies of *G. fasciventris* and *A. orientalis* on BaMV-free bamboo seedlings in 200-mesh insect domes. The insects and the bamboo seedlings were indexed bi-weekly to ensure the BaMV-free status (data not shown). These BaMV-free dipteran insects were used in the following assays.

## BaMV Could be Ingested into the Bodies of the Dipteran Insects

To explore the relationship between BaMV and the dipterans, GFP-tagged BaMV virions were purified from *C. quinoa* leaves inoculated with pCB-GFP2a-CP (**Figure 3A**), which express

GFP-fused BaMV CP. Examination of the purified GFP-tagged BaMV virions under UV illumination confirmed the presence of GFP-tags on the purified virions (**Figure 3B**, tube on the right), as compared to the wild type BaMV virions (**Figure 3B**, tube on the left). The dipterans were fed with liquid medium containing 0.1 mg/ml of purified GFP-tagged or wild type BaMV virions (**Figure 3C**) in an inverted 50-ml Falcon conical tube for 24 h. The presence of GFP signals (likely representing the GFP-tagged BaMV virions) in dipterans were examined by using a LAS-4000 Chemiluminescence and Fluorescence Imaging System (Fujitsu Life Sciences, Tokyo, Japan). The result revealed that green fluorescence was clearly visible in the abdomen portion of *G. fasciventris* fed with GFP-tagged BaMV virions (**Figures 3D,E**, lower row), while those fed with wild type BaMV exhibited only background fluorescence. However, the green fluorescence might also be contributed by the unassembled GFP-tagged BaMV CP or GFP-2a proteins in the virion preparations, thus the presence of BaMV RNAs in the dipterans were further assayed. Following surface decontamination by RNase Away and RNA extraction,

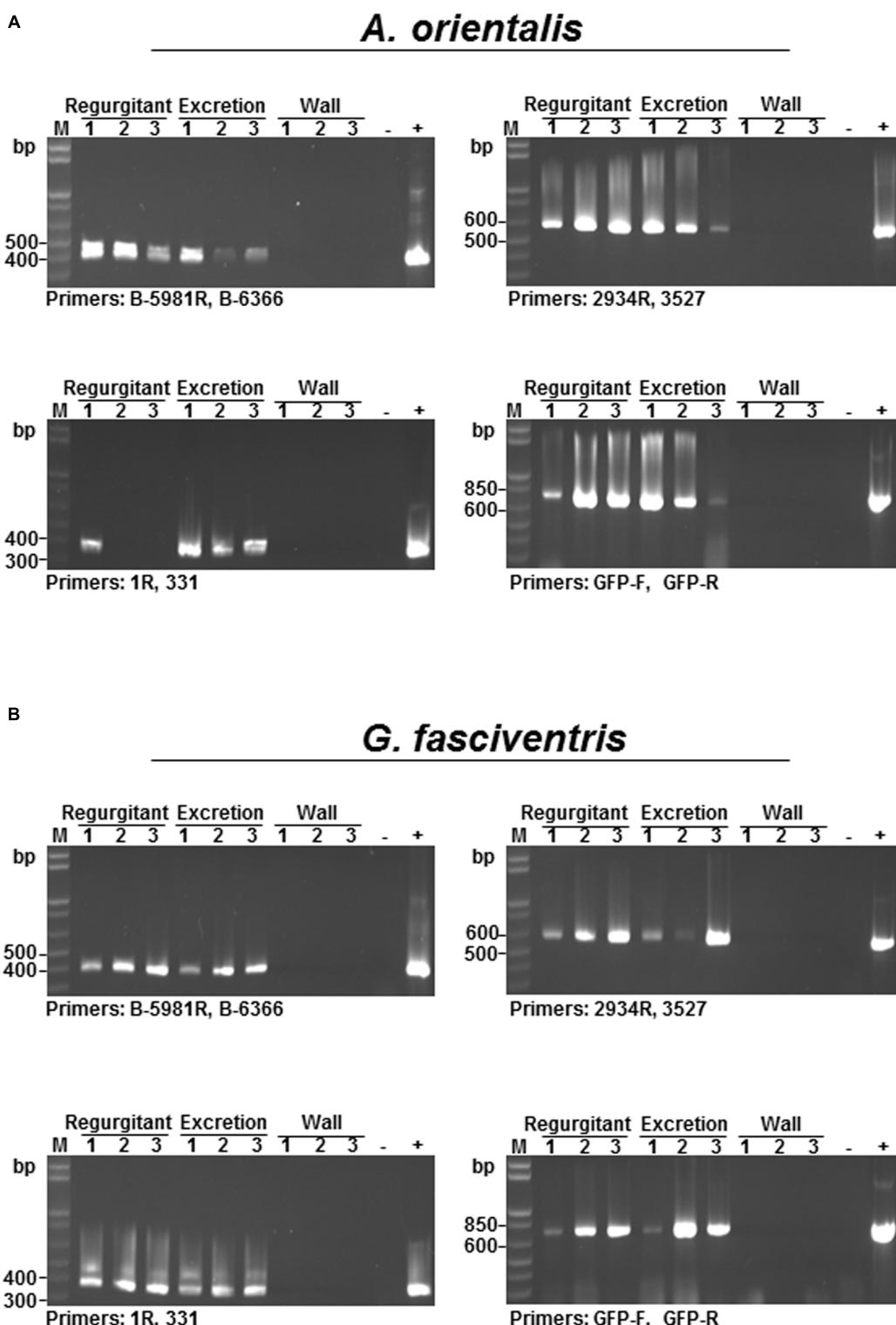


**FIGURE 4 | Verification of the internal localization of BaMV in the dipterans.** Following feeding with BaMV-containing medium for 24 h and virus-free medium for 72 h, the presence of BaMV RNA on the external surfaces (lanes labeled “External”) and within insect bodies (lanes labeled “Internal”) of individual *A. orientalis* (**A**) and *G. fasciventris* (**B**) flies were assayed by RT-PCR as described above. RT-PCR products using nucleic acids extracted from insects fed on healthy bamboo plants or BaMV-containing medium (1  $\mu$ l) as the templates were used as negative (lanes –) and positive controls (lanes +), respectively. (**C**) Detection of the retention of BaMV within dipteran insects by RT-PCR. The presence of BaMV RNA within *G. fasciventris* samples fed with GFP-tagged BaMV was examined at two-week intervals (indicated on top of the lanes) by RT-PCR as described above (four insects per group). Lanes A1 and A2, *A. orientalis* samples collected at 4 weeks post BaMV-feeding; lane M, size marker, lane +, positive control.

the presence of BaMV genomic RNA in *A. orientalis* and *G. fasciventris* fed with BaMV-containing medium was confirmed by RT-PCR (**Figure 3F**). The observations suggested that BaMV may actually enter the digestive systems of dipterans, instead of just temporarily associated with the mouthparts of the insects as in the cases for non-persistent type transmission (Ng and Falk, 2006).

### BaMV May Be Retained Inside the Bodies of *A. orientalis* and *G. fasciventris*

To verify the localization of BaMV on the dipterans, *A. orientalis* and *G. fasciventris* insects were fed with BaMV-containing medium for 24 h as described above, followed by virus-free medium for 72 h. The flies were collected individually in 1.5-ml Eppendorf tubes, and then soaked in nucleic acid extraction



**FIGURE 5 | Detection of GFP-tagged BaMV in the regurgitant and excretion fluids of dipteran insects.** To investigate whether BaMV RNA could pass through the digestive systems of diptera insects, the regurgitant and excretion fluids (as indicated on the top of the lanes) were collected from *A. orientalis* (**A**) and *G. fasciventris* (**B**) fed with GFP-tagged BaMV-containing media and subjected to RT-PCR analysis for the presence of BaMV RNA, using different primer pairs (as indicated at the bottom of each gel). To test whether the walls of the feeding tube were contaminated during the feeding process, aliquots of nucleic acid extraction buffer (2  $\mu$ l) were used to elute materials surrounding the regurgitant or excretion droplets, and subjected to RT-PCR analysis concurrently (lanes labeled "Wall"). For the positive control, 1  $\mu$ l of GFP-BaMV-supplemented medium was assayed concurrently. Lane M, size marker.

buffer (120  $\mu$ l) with gentle shaking (60 rpm) for 3 min to elute the virus particles possibly adhered to the external surfaces. The eluate was then collected and subjected to total nucleic acid extraction as described above. The washed insect bodies were centrifuged twice at 1000  $\times$  g for 10 s to remove excess wash buffer, and also subjected to total nucleic acid extraction. Total nucleic acids from insects fed on healthy plants were used as the templates for the negative controls. The presence of BaMV genomic RNAs was assayed by RT-PCR using primer pair B-5981R and B-6366. The result revealed that BaMV RNAs could only be detected in the insect bodies, not in the eluates of the external portions of *A. orientalis* and *G. fasciventris* (Figures 4A,B, respectively). The above observations suggested that BaMV virions are not simply adhering to the surfaces of *A. orientalis* and *G. fasciventris* for dispersion by the mechanical contacts between insects and plants. To test the ability of the dipterans to retain BaMV RNAs, the dipterans were fed with BaMV-containing medium for 24 h, followed by feeding with virus-free medium for up to 6 weeks. Total RNAs were extracted from samples collected at 2-week intervals and subjected to RT-PCR analysis for the presence of BaMV RNA. The results showed that BaMV RNAs could be retained by *G. fasciventris* for up to 4 weeks (Figure 4C). On the other hand, the BaMV RNA appeared to be degraded after 4 weeks in *A. orientalis*, but barely detectable amount of RT-PCR products was still observed at least in one of the samples (Figure 4C, lane A1).

## BaMV May Survive the Digestive Systems of the Diptерans

For a virus to be transmitted by an insect vector following ingestion into the abdomen, the virus must survive and pass through the digestive system of the insect. When dipterans feed, they constantly regurgitate crop fluids with digestive enzymes to help dissolve the substrate (Coronado-Gonzalez et al., 2008). The excretions are regularly deposited around the feeding sites. Therefore, the presence of BaMV RNAs in the regurgitants and excretions were examined by RT-PCR to test the survival of BaMV RNA through the digestive system. *A. orientalis* and *G. fasciventris* adults were starved for 4 h, and then fed with media containing GFP-tagged BaMV in 50-ml Falcon conical tubes for 24 h. The regurgitants and excretions of the flies on the wall of the tubes were collected by dissolving in nucleic acid extraction buffer (2  $\mu$ l) under a dissecting microscope, and subjected to RNA extraction and RT-PCR analysis as described above. Although it has been shown that BaMV RNA could not be detected on

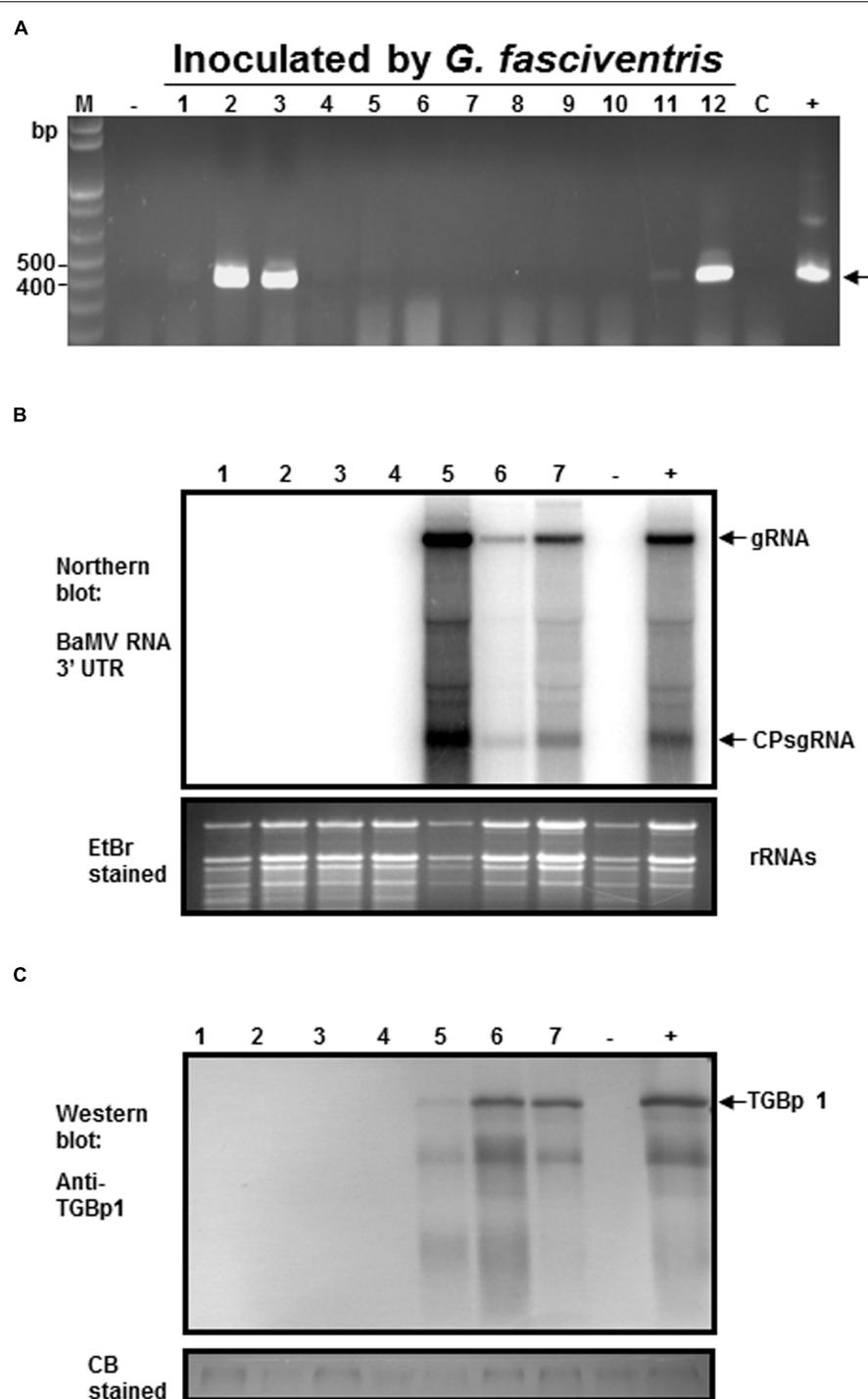
the external surfaces of these insects (Figures 4A,B), there is still the possibility that the walls of the tubes could be contaminated by GFP-tagged BaMV from the evaporation of the medium or the streaking of the insects. To test the possibility of contamination on the walls, aliquots of 2- $\mu$ l nucleic acid extraction buffer were used to elute any possible contaminants present on the areas surrounding the regurgitant and excretion droplets. The eluates (lanes labeled "Wall" in Figure 5) were then subjected to nucleic acid extraction and RT-PCR analysis concurrently with the regurgitant and excretion samples, serving as negative controls. To overcome the problem of relative inefficient amplification of full-length GFP-tagged BaMV RNAs (~7.0 kb) directly by RT-PCR using primers 1R and oligo dT, an alternative approach was adopted by using different primer pairs (as shown in Figure 3A) to detect different portions of the GFP-tagged BaMV RNA throughout the entire length, using the first-strand cDNA synthesized using the oligo-dT primer as the only template. Since the first strand cDNA is synthesized using oligo-dT primer starting from the 3'-poly(A) tail of BaMV genome, the amplification of the different fragments, especially the one corresponding to the very 5' terminus (amplified by primers 1R and 331), indicated that the intact BaMV genomic RNA was present in the sample. The results revealed that intact BaMV RNAs could survive the digestive enzymes in the regurgitants and pass through the digestive system to reach the excretion portion of both *A. orientalis* and *G. fasciventris* (Figure 5, lanes labeled "Regurgitant" and "Excretion"). In contrast, no BaMV RNA was detected in the eluates from the wall areas surrounding the regurgitant and excretion droplets (Figure 5, lanes labeled "Wall"). The above observations raised the possibility that BaMV might be transmitted by the dipterans through the regurgitants or excretions while probing and feeding on bamboos.

## *A. orientalis* and *G. fasciventris* Could Mediate the Transmission of BaMV to Bamboo Seedlings

To test the abilities of the dipterans in transmitting BaMV among bamboo plants, the following experiments were conducted. To simulate the possible insect-mediated transmission during harvesting process, the newly emerged bamboo shoots were cross-sectioned at the crowns to create wounds for insect-mediated virus transmission assays. Since the fusion of the GFP at the N-terminus of BaMV CP interfered with the infectivity in bamboos, the dipterans were fed with liquid media containing purified wild type BaMV virions (0.1 mg/ml) for 24 h, and then subjected to inoculation assays by co-incubation with the bamboo seedlings with cross-sectioned shoots in 200-mesh insect domes. Within each dome, four to seven *A. orientalis* or *G. fasciventris* fed with liquid media containing wild type BaMV were used to inoculate the bamboo seedlings. The dipterans were allowed to feed freely for 24 h, and then removed manually. The newly emerged leaves from the new shoots of the inoculated bamboo seedlings were assayed for the presence of BaMV RNA by RT-PCR at day 60 post inoculation.

**TABLE 3 | Summary of insect transmission assays on bamboos.**

| Experiment | Bamboo seedling type | Dipteran insect species | # BaMV-infected/# tested plants |
|------------|----------------------|-------------------------|---------------------------------|
| 1          | Air-layering         | <i>G. fasciventris</i>  | 1/6                             |
| 2          | Tissue-cultured      | <i>G. fasciventris</i>  | 2/12                            |
| 3          | Air-layering         | <i>A. orientalis</i>    | 2/14                            |
| 4          | Air-layering         | <i>G. fasciventris</i>  | 5/12                            |



**FIGURE 6 | Analysis of the *G. fasciventris*-mediated BaMV transmission by RT-PCR. (A)** BaMV-free bamboo seedlings were inoculated by *G. fasciventris* fed with BaMV-containing medium in the insect domes. Samples were collected from the newly emerged leaves at the 60th day post inoculation (dpi), and subjected to total nucleic acid extraction and RT-PCR analysis as described above. Lane M, size marker; lane C, sample of cotton balls used for the feeding of the dipterans at the beginning of the experiment; lanes – and +, negative and positive controls as described in **Figure 2**. To verify the inoculation results, northern (**B**) and western blot (**C**) were performed using  $^{32}\text{P}$ -labeled probes specific for BaMV RNA 3' untranslated region (Hsu et al., 2000) or antibody against TGBp1 (Hung et al., 2014). Lanes 1–4, RNA or protein extracts of the un-inoculated siblings from the same mother-stock of the bamboo seedlings tested positive in (**A**); lanes 5–7, RNA or protein extracts from newly emerged leaves of seedlings tested positive for BaMV in panel A (samples #2, #3 and #12, respectively). Lanes – and +, samples from healthy and BaMV-infected bamboo plants, respectively. The positions of BaMV genomic RNA (gRNA), CP subgenomic RNA (CP sgRNA), and TGBp1 are indicated by the arrows.

The results of dipteran insect-mediated BaMV transmission assays were summarized in **Table 3**. The representative result (**Figure 6A** from Experiment 4 in **Table 3**) demonstrated that *G. fasciventris* may actually mediate the transmission of BaMV to bamboo seedlings. To test the possibility of BaMV contamination in the tested plants before the inoculation, we have traced back and examined the un-inoculated siblings from the same mother-stock of the seedlings which tested positive for BaMV after the completion of Experiment 4 (**Table 3**) by northern (**Figure 6B**) and western blot (**Figure 6C**) analyses using <sup>32</sup>P.-labeled probe specific to the 3' untranslated region of BaMV RNA (Hsu et al., 2000) and antiserum specific to TGBp1 (Hung et al., 2014), respectively. Three of the inoculated bamboo seedlings (**Figure 6A**, lanes 2, 3, and 12) which tested positive for BaMV in Experiment 4 (**Table 3**) were also assayed concurrently for the products of BaMV infections in the newly emerged leaves. As shown in **Figures 6B,C**, the un-inoculated siblings from the same mother stock (lanes 1–4) remained BaMV-free, while the subgenomic RNAs and TGBp1 protein, which are produced only after the successful infection of BaMV, could be detected in those tested positive (lanes 5–7) in the insect-mediated inoculation assays (**Figure 6A**, lanes 2, 3, and 12).

## DISCUSSION

### A Novel Finding That Dipterans May Mediate the Transmission of BaMV, a Potexvirus, among Bamboos

Based on the field observations, we explored the possibility of insect-mediated transmission of BaMV among bamboos in this study. Several lines of evidence were provided to support the notion that dipterans may serve as the vector for BaMV, including the association of BaMV with the internal portion of the dipterans, the survival of BaMV RNA through the digestive systems, and the inoculation assays on bamboo seedlings. Our study revealed a novel finding with regards to the role of dipterans as vectors for a plant virus, and the insect-mediated transmission of a potexvirus.

For the role of dipterans as vectors for a plant virus, the flies have been known to transmit many animal pathogens, including viruses (see Baldacchino et al., 2013 for an excellent review), but, to our knowledge, the transmission of a plant virus by dipteran insects with sponge-like mouthparts has not been reported previously. As vectors for animal pathogens, dipterans is believed to transmit viruses by a “mechanical mode,” which appears to occur through either contamination of mouthparts or regurgitation of the contents of the digestive systems onto the openings or wounds of the animals (Baldacchino et al., 2013). This mode of transmission appears to be suitable for plant viruses, especially for those with highly stable virions such as *Tobacco mosaic virus* or *Potato virus X*. However, to our knowledge, no dipterans with sponge-like mouthparts have been reported to transmit plant viruses prior to this study.

As for the insect-mediated transmission of a potexvirus, most potexviruses are not known or thought to be transmitted by insect vectors (Koenig and Lesemann, 1978; Jelkmann et al., 1990). Recent reviews on the insect-transmission of plant viruses did not consider potexviruses in the discussions (e.g., Blanc et al., 2014; Whitfield et al., 2015). In an earlier review (Ng and Falk, 2006), humans are listed as the only rare animal vector for potexvirus transmission. However, *Potato virus X* and *White clover mosaic virus* has been reported to be transmitted by grasshoppers and aphids, respectively (Schmutterer, 1961; Goth, 1962). It has also been reported that *Potato aucuba mosaic virus* could be transmitted by aphids in the presence of a helper virus in the genus *Potyvirus* (Kassanis and Govier, 1971). In addition, the transmission of SMYEA V, by aphids in a persistent mode has been reported (Jelkmann et al., 1990), although it is possible that an SMYE associated luteovirus reported previously (Yoshikawa et al., 1984; Martin and Converse, 1985; Spiegel et al., 1986) may serve as the helper virus for heterologous encapsidation and aphid transmission, or SMYEA V may be aphid-transmitted by other unknown mechanism (Jelkmann et al., 1990). Nevertheless, the transmission of potexviruses by dipterans with sponge-like mouthparts has not been known previously. The findings in this study thus revealed a novel role of the dipterans with sponge-like mouthparts as a vector for a plant virus, at least for a potexvirus, BaMV.

### Relationship between BaMV and Dipterans

The results in this study also provide information for dissecting the relationship in transmission between BaMV and the dipterans. Based on the transmission characteristics, the insect transmission modes of plant viruses have been practically categorized as “non-persistent,” “semi-persistent,” “persistent-circulative,” and “persistent-propagative,” etc. (Ng and Falk, 2006). In this study, we demonstrated that BaMV may actually be ingested into the abdomen (**Figures 3D–F**), remain associated with the dipterans for up to 4 weeks, and survive in the regurgitants and excretions (**Figures 4, 5**). These results suggested that the relationship between BaMV and these dipterans could be classified as being semi-persistent or persistent (Ng and Falk, 2006). However, it remains unknown whether BaMV may circulate within the hemolymph of the flies and finally into the salivary gland to be regurgitated, or whether BaMV could replicate within the dipterans.

Female phytophagous flies are known to feed by piercing the surface of plants by the ovipositors (as seen on the posterior portion of *G. fasciventris* in **Figure 1C**), then sucking the fluids without laying eggs. During the probing and feeding process, the dipterans with sponge-like mouthparts would regurgitate crop fluid and deposit excretions around the feeding sites (Coronado-Gonzalez et al., 2008). The digestive enzymes in the regurgitants or the excretions may assist in the penetration of cell walls or membranes to facilitate the infection of bamboo tissues by BaMV. This may be one of the possible scenarios how dipterans

mediate the transmission of BaMV to bamboos. However, the low infection efficiency in the dipteran insect-mediated transmission assays (14–41%, **Table 3**) and the involvement of artificially created wounds in the assays suggested that the dipteran insects may transmit BaMV in a mechanical-like manner, not directly feeding BaMV into the plants. Further studies are required to fully analyze the relationship between BaMV and the dipterans and the mode of transmission involved.

## The Impact on Current Integrated Pest Management System for BaMV Disease in Bamboos

As mentioned above, no known insect vectors have been reported for BaMV (Elliot and Zettler, 1996; Hsu and Lin, 2004; Nelson and Borth, 2011), thus the current recommendation for the management of BaMV in bamboos did not include the control and prevention of dipterans. The present production systems of BaMV-free bamboo seedlings in Taiwan use indexed bamboo seedlings originated from meristem-tip tissue cultures (Hsu et al., 2000), but the amplification of the original BaMV-free seedlings for downstream growers is dependent on the seedling nurseries in the fields.

The finding in this study that dipterans may mediate the transmission of BaMV highlights the importance of the integration of dipteran insect control and prevention measures into the current disease management system against BaMV. Since the dipterans tested in this study exhibited much longer virus acquisition feeding time, virus retention time, and transmission feeding time, compared to those of the non-persistent mode of transmission, the transmission mode of BaMV by the dipterans may be categorized as at least “semi-persistent,” if not “persistent.” Thus, the dipterans may be controlled by using suitable pesticides, without the concern of causing increased dispersals of the viral diseases as seen for “non-persistent” mode of transmission.

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## CONCLUSION

To our knowledge, this is the first report describing the transmission of a plant virus by dipterans with sponge-like mouthparts. These results expanded the types of insects as vectors for plant viruses, and suggested that dipteran insect control should be concerned and integrated into the disease management measures against viruses, such as BaMV, that are structurally stable enough to survive through the digestive systems of these insects.

## AUTHOR CONTRIBUTIONS

Study conception and design: Y-HH, N-SL, and C-CH. Acquisition of data: K-CC, L-TC, Y-WH, Y-CL, C-WL, J-TL, and C-CH. Analysis and interpretation of data: Y-HH, N-SL, Y-WH, and C-CH. Drafting and critical revision of the manuscript: Y-HH, N-SL, Y-WH, and C-CH.

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**Conflict of Interest Statement:** 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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# Phylogeography and Coevolution of Bamboo Mosaic Virus and Its Associated Satellite RNA

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*Bamboo mosaic virus* (BaMV), a plant potexvirus, has been found only in infected bamboo species. It is frequently associated with a large, linear single-stranded satellite RNA (satBaMV) that encodes a non-structural protein. Decades of collecting across a wide geographic area in Asia have accumulated a sizable number of BaMV and satBaMV isolates. In this study, we reconstructed the BaMV phylogeny and satBaMV phylogeny with partial coat protein gene sequences and partial genomic sequences, respectively. The evolutionary relationships allowed us to infer the phylogeography of BaMV and satBaMV on the Asian continent and its outlying islands. The BaMV phylogeny suggests that the BaMV isolates from Taiwan, unsurprisingly, are most likely derived from China. Interestingly, the newly available satBaMV isolates from China were found to be most closely related to the previously established Clade III, which is found in India. The general pattern of clustering along the China/India and Taiwan divide led us to hypothesize that the Taiwan Strait has been a physical barrier to gene flow in the past evolutionary history of both BaMV and satBaMV. Lastly, cophylogeny analyses revealed a complex association pattern between BaMV and satBaMV isolates from China. In general, closely related BaMV sequences tend to carry closely related satBaMV sequences as well; but instances of mismatching with distantly related satBaMV isolates were also found. We hypothesize plausible scenarios of infection and superinfection of bamboo hosts that may be responsible for the observed association pattern. However, a more systematic sampling throughout the geographic distribution of various bamboo species is needed to unambiguously establish the origin, movement, and evolution of BaMV and satBaMV.

**Keywords:** BaMV, satBaMV, phylogeography, cophylogeny, evolution

## INTRODUCTION

Population genetic surveys and phylogenetic reconstructions of plant viruses and their associated satellites offer us invaluable insights into not only the observed patterns and inferred processes of plant virus evolution (García-Arenal et al., 2003) but also the ecological interactions that may contribute to long-distance dispersal and emergence of new diseases (Fargette et al., 2006). Ever since the enigmatic RNA 5 was confirmed to be a satellite RNA (satRNA) associated with the *Cucumber mosaic virus* (CMV) (Kaper et al., 1976), this model system has become the most widely studied combination of helper virus and satRNA. Recent works span their basic biology

(Palukaitis et al., 1992; Roossinck et al., 1992; García-Arenal and Palukaitis, 1999; Palukaitis and García-Arenal, 2003; Jacquemond, 2012), their ecology (Gallitelli, 2000) and evolution (Roossinck, 2001, 2002). Especially interesting is a series of field surveys and subsequent experimental studies that investigate a multi-year epidemic of CMV and its associated satRNAs (satCMV) in Eastern Spain (Fraile and García-Arenal, 1991; Jordá et al., 1992; Aranda et al., 1993; Alonso-Prados et al., 1998; Escriu et al., 2000a,b; Betancourt et al., 2011). A multi-year epidemic ravaged by CMV and satCMV in Italy yielded a similar pattern of genetic variation in the field (Grieco et al., 1997). Various phylogenetic and phylogeographic studies of plant viruses and their associated satellites in nature have revealed clustering of isolates based on symptoms, plant hosts, and/or geographic origins (Grieco et al., 1997; Cabrera et al., 2000; Abubakar et al., 2003; Pinel et al., 2003; Fargette et al., 2004; Tomitaka and Ohshima, 2006; Olarte Castillo et al., 2011; Venkataravannappa et al., 2011; Cuevas et al., 2012a,b). However, it is to be noted that most of these studies are with viruses that adopt the acute lifestyle (Roossinck, 2010), infecting plant hosts that are predominantly annual crops.

Bamboos are a group of evergreen perennial grasses belonging to the grass family Poaceae (Kelchner and Bamboo Phylogeny Group, 2013; Clark et al., 2015). There are approximately 1,400 to 1,500 bamboo species, distributed worldwide, except Europe and Antarctica (Kelchner and Bamboo Phylogeny Group, 2013; Clark et al., 2015). Of the two main bamboo types, the most economically, ecological, and culturally important, and also what we are most familiar with, is the woody bamboos (as opposed to the herbaceous bamboos). Of the reported bamboo diseases, only two are caused by viruses (Su and Wang, 2015). *Bamboo mosaic virus* (BaMV) was first recognized in Brazil in 1974 in two bamboo species, *Bambusa multiplex* and *B. vulgaris* (Lin et al., 1977). Besides South America, BaMV has subsequently been reported in various parts of the world, including North America, Asia, and Australia (Lin et al., 1992, 1993, 1995, 2015; Thomas and Dodman, 1999; Nelson and Borth, 2011). Curiously, no case of BaMV infection has been reported on the African continent, where many bamboo species are found (Clark et al., 2015). It should not come as a surprise if BaMV is eventually reported in Africa as well.

*Bamboo mosaic virus* belongs to the genus *Potexivirus* of the family *Alphaflexiviridae* (Adams et al., 2011). It is a flexuous rod of approximately 490 nm × 15 nm in size (Lin et al., 1977; DiMaio et al., 2015). Its single-stranded RNA genome is approximately 6,400 nucleotides long, encoding a polymerase, triple-gene block (proteins involved in cell-to-cell movement), and a coat protein (CP) (Lin et al., 1992, 1994). In the field, BaMV is frequently associated with a satellite RNA (satBaMV) (Lin and Hsu, 1994; Yeh et al., 2004; Wang et al., 2014). Although the genome of the type member, satBaMV-BSF4, is only 836 nucleotides long, it nevertheless belongs to a group of large linear single-stranded satellite RNAs (satRNAs) (Mayo, 1991; Briddon et al., 2012). However, unlike most other members of this satRNA group, all of which are all associated with helper viruses in the family *Secoviridae*, satBaMV is the only known example of satRNA associated with a member in the family *Alphaflexiviridae*.

(Mayo, 1991; Briddon et al., 2012). As is typical of this large satRNA group, satBaMV also encodes a non-structural protein, P20, which is involved in the systemic movement of satBaMV within the infected host (Lin et al., 1996; Palani et al., 2006, 2012; Chang et al., 2016). The P20 protein is the only known large satRNA-encoded protein not required for satRNA replication (Lin et al., 1996).

Since satBaMV depends on its helper virus for genome replication, cell-to-cell movement, and encapsidation, it can be viewed as a molecular parasite exploiting various vital functions of its host BaMV (Nee, 2000). That is, interaction between the help virus and its associated satRNA is typically seen as antagonistic, presumably via competition for accessing the viral encoded RNA-dependent RNA polymerase (RdRp) for replication (Wu and Kaper, 1995). For BaMV and satBaMV, the competition for RdRp is likely mediated through the untranslated 5'-end regions of their genomes (Chen et al., 2007, 2010, 2012). One of the consequences of such an interaction is manifested as the severity of the infected plant hosts' symptoms (Collmer and Howell, 1992). BSF4 and BSL6 are two of the most frequently studied satBaMV isolates. Their disease symptoms, when coinfecting with BaMV separately, represent the opposite ends of the spectrum, with BSF4 infection showing a severe mosaic symptom, while BSL6 infection a relatively mild one (Hsu et al., 1998). This apparent antagonistic interaction, albeit manifested with a wide range of symptom severity, is further complicated by other indirect interactions mediated through the defense mechanisms of the host plant (Hu et al., 2009). Consequently, the coevolutionary patterns may be host plant dependent. One possible consequence of long-term interaction is the evolution of helper virus specificity by the satRNA. For example, some large *Nepovirus*-associated satellite RNAs can be supported only by certain isolates or serotypes of their helper viruses (Murant and Mayo, 1982; Roossinck et al., 1992; Fritsch et al., 1993; Oncino et al., 1995). In the most extreme case, the specificity can simply be determined by the presence or absence of a single amino acid residue (Roossinck et al., 1997). But it should be noted that there is no solid empirical demonstration for the hypothesized arms race dynamics between isolates of BaMV and satBaMV, and consequently it is not clear to what extent these interactions are a driving force for sequence evolution.

In contrast to well-established plant virus systems, BaMV has not been subjected to phylogeographic investigation. However, limited studies with full-length genomic sequences do reveal a clustering pattern, and suggest that isolates from Taiwan are derived from China (Lin et al., 2016, 2017b). In comparison, we have previously reported a more extensive study on various satBaMV isolates sampled from parts of China, India, and Taiwan (Wang et al., 2014). Phylogenetic analysis uncovered three distinct and well-supported satBaMV clades that most likely have persisted for many decades, if not longer. Interestingly, there is no single characteristic, such as geographic origin or host bamboo species, defining these clades. For example, Clade I, exemplified by the type sequence BSF4, is composed of isolates from various BaMV-infected bamboo species on Taiwan and the Hainan Island of China and also, interestingly, one single sample from the United States. Isolates in Clade II, e.g., the type sequence BSL6,

are almost exclusively found in Ma bamboo (*Dendrocalamus latiflorus* Munro) on Taiwan. Clade III is currently found only in India infecting *B. vulgaris*. As more samples from different geographic locations and host bamboo species are included in the analysis, it is not clear whether these three satBaMV clades will persist.

In this study, we take advantage of newly available BaMV and satBaMV sequences from China that were isolated from various bamboo species in three botanical garden settings (Lin et al., 2015, 2016, 2017a). We investigate how BaMV and satBaMV may have migrated across the Asian continent. We also hypothesize the mechanisms responsible for the observed cophylogeny pattern between BaMV and satBaMV.

## MATERIALS AND METHODS

### Sequence Acquisition

We used sequence information of BaMV and its associated satBaMV from three sources: (1) novel sequences from China deposited at the GenBank (accession numbers KP233222 and KP256025-256071 for BaMV, and KP233223 and KP256110-KP256146 for satBaMV), (2) sequences from our previous studies (Yeh et al., 2004; Wang et al., 2014), and (3) BaMV CP gene sequences from Taiwan that are new to this study. In our previous study (Wang et al., 2014), a total of 568 sequences, most of which from Taiwan, were used to reconstruct the satBaMV tree. To facilitate phylogenetic reconstruction and to avoid over-representation of satBaMV sequences from Taiwan, we selected five sequences from each of the six groups (*B. oldhamii*, *B. vulgaris*, *D. latiflorus*, and *D. latiflorus* cv. Mei-nung from Taiwan; *B. ventricosa* from Hainan Island, China; and *B. vulgaris* from India) for this study. The goal of the selection is to have maximal diversity represented for each group. The selection is guided by within-group pairwise comparisons using MEGA7 v7.0.20 (Kumar et al., 2016). The new BaMV sequences from Taiwan were isolated in 1994–2000, from various infected bamboo species and locations (Yeh et al., 2004). BaMV virions from infected bamboo leaves were purified and the viral RNA extracted as described previously (Lin and Chen, 1991). Purified viral RNA was used as the template and oligonucleotides B81 (5'-ACGGGGAGCTT<sub>20</sub>-3', underlined letters indicate the SacI site) as the primer for reverse transcription to synthesize the first strand cDNA. The primer pair of B81 and B43 (5'-CGACGTTTGGAAATAATAAAC-3', underlined letters indicate the BstXI site), which complements the 5' flanking promotor region of the CP gene, were used to amplify the CP gene. The DNA amplicon was separated with 1% agarose gel, gel-purified, ligated into pGEM-T Easy cloning vector (Promega, Madison, WI, United States), and then transformed into *Escherichia coli* DH5 $\alpha$ . The resulting plasmid, carrying the inserted sequence, was selected and sequenced. DNA sequencing was performed by ABI 377A Sequencer using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, United States).

All new sequences are deposited at the GenBank. Supplementary Material S1 lists all sequences used in this

study, including isolate names, locations and dates of sampling, and GenBank accession numbers. Supplementary Material S2 shows the map with the approximate sampling locations.

### Sequence Alignment

The recently available data from China are partial sequences of BaMV CP genes and partial genomic sequences of satBaMV. For sequence alignment, all other sequences were trimmed to the same lengths as those from China. To avoid confusion and to facilitate orientation, the BaMV-S and satBaMV-BSF4 genomic sequences, accession numbers AF018156 and AY205227, respectively, are used as references for positioning the nucleotide and protein sequences used in this study. For BaMV, the nucleotide sequences corresponding to the BaMV-S genomic sequence, nucleotides 5611–6138 (528 nucleotides; 72.4% of the CP gene sequence; encoding amino acid residues 39–213), are used. For satBaMV, the nucleotide sequences corresponding to the satBaMV-BSF4 genomic sequence, nucleotides 271–742 (472 nucleotides; 56.5% of the genomic sequence; encoding P20 amino acid residues 38–183), are used.

The most closely related sequences to BaMV CP and satBaMV P20 proteins are the CP proteins of the *Foxtail mosaic virus* (FoMV, GenBank accession number NC\_001483) (Yamaji et al., 2001) and the *Panicum mosaic satellite virus* (SPMV, GenBank accession number NC\_003847) (Liu and Lin, 1995), respectively. Therefore, these two sequences were used as outgroups to root the corresponding BaMV and satBaMV trees. Two pairs of full-length protein sequences, FoMV CP/BaMV-S CP and SPMV CP/satBaMV-BSF4 P20, were aligned separately using Expresso of the T-coffee online service<sup>1</sup> (Notredame et al., 2000; Armougom et al., 2006; Di Tommaso et al., 2011). The partial BaMV and satBaMV nucleotide sequences were aligned using the online service, Clustal Omega<sup>2</sup> (Sievers et al., 2011). The initial nucleotide sequence alignments were then manually adjusted using the protein sequence alignments as guides.

### Phylogenetic Reconstruction

We used both the Bayesian inference (BI) and maximum likelihood (ML) methods to infer the relationships among the aligned BaMV and satBaMV sequences. For each method, alignments with or without the outgroup sequences were also constructed. The substitution model “GTR+G+I,” selected by the automatic model selection function of the PhyML server version 3.0<sup>3</sup> (Guindon et al., 2010), was used for all tree constructions, except for that of the BaMV ML tree with FoMV as the outgroup, for which the model “GTR+G” was used. For the BI method, MrBayes (Huelsenbeck and Ronquist, 2001), version 3.2 (Ronquist et al., 2012) running on Mac OS X version 10.12, was used. All reconstructions with MrBayes were run for 10,000,000 generations and had 25% burn-in. The convergence of the runs was assessed using the program Tracer v1.6 (Rambaut et al., 2014). For all runs, the ESS (effective sample size) values for the log likelihood LnL ranged from

<sup>1</sup><http://tcoffee.crg.cat/apps/tcoffee/do:expresso>

<sup>2</sup><http://www.ebi.ac.uk/Tools/msa/clustalo/>

<sup>3</sup><http://www.atgc-montpellier.fr/phym/>

1519 to 3196, and the PSRF (potential scale reduction factor) values were all close to 1.0. For the ML trees, we used the online PhyML server (see above). A bootstrap of 500 replicates was used to estimate branch support. We used the program TreeGraph 2 (Stöver and Müller, 2010) to collapse branches with low support values. For the BI trees, the criterion of branch collapsing is for the posterior probability  $<0.90$ , and for the ML trees the criterion is replicate number  $<375$  (75%). The program FigTree, version 1.4.3 (Rambaut, 2012), was used to visualize and manipulate the trees, including the function of midpoint rooting.

## Cophylogeny Analysis

Both *Jane* version 4.0.1 (Conow et al., 2010) and *PACo* version 1.1.r (Balbuena et al., 2013) were used for cophylogeny analysis. For analysis using *Jane*, we employed default costs of “cospeciation” = 0, “duplication” = 1, “duplication and host switch” = 2, “loss” = 1, and “failure to diverge” = 1. We found that the values for the Genetic Algorithm parameters have some effects on the estimated minimum cost. We conducted an initial exploration of the parameter space of the “number of generations” and “population size” by varying their values from the default of 100 to a range of 30 to 1,500 to obtain the corresponding range of the estimated minimum costs. All estimated costs ranged from 45 to 52. Because the higher the values are the longer it takes to complete a single simulation, therefore we used the values of 50 and 1,500 for “number of generations” and “population size,” respectively, as a compromise between the precision of cost estimation and the time to complete the subsequent randomizations. We used 100 randomizations to obtain the cost distribution of randomized associations. The *PACo*, version 1.1, is an R script and the analysis was conducted using RStudio (v. 0.99.903) (RStudio Team, 2012) running on top of R (v. 3.3.1) (R Core Team, 2016). We used the *cophylo* function from the *phytools* package (Revell, 2012) to visualize associations between BaMV and satBaMV isolates from China.

The input files for these two analyses are based on the collapsed Bayesian trees shown in **Figures 1, 2**, although they are not the same. For the *PACo* analysis, which requires the information on branch length, the original tree files from **Figures 1, 2** were used. For the *Jane* analysis, which requires each “parasite species” to have at least one corresponding “host species,” so the tree file for **Figure 2** is not suitable for the analysis. To circumvent this problem, the tree topologies of the 38 Chinese BaMV and satBaMV isolates were manually extracted from **Figures 1, 2** to be used as the input files for the *Jane* analysis. The *cophylo* plot in **Figure 3** also used the same input file used in the *Jane* analysis to simplify the presentation.

## RESULTS AND DISCUSSION

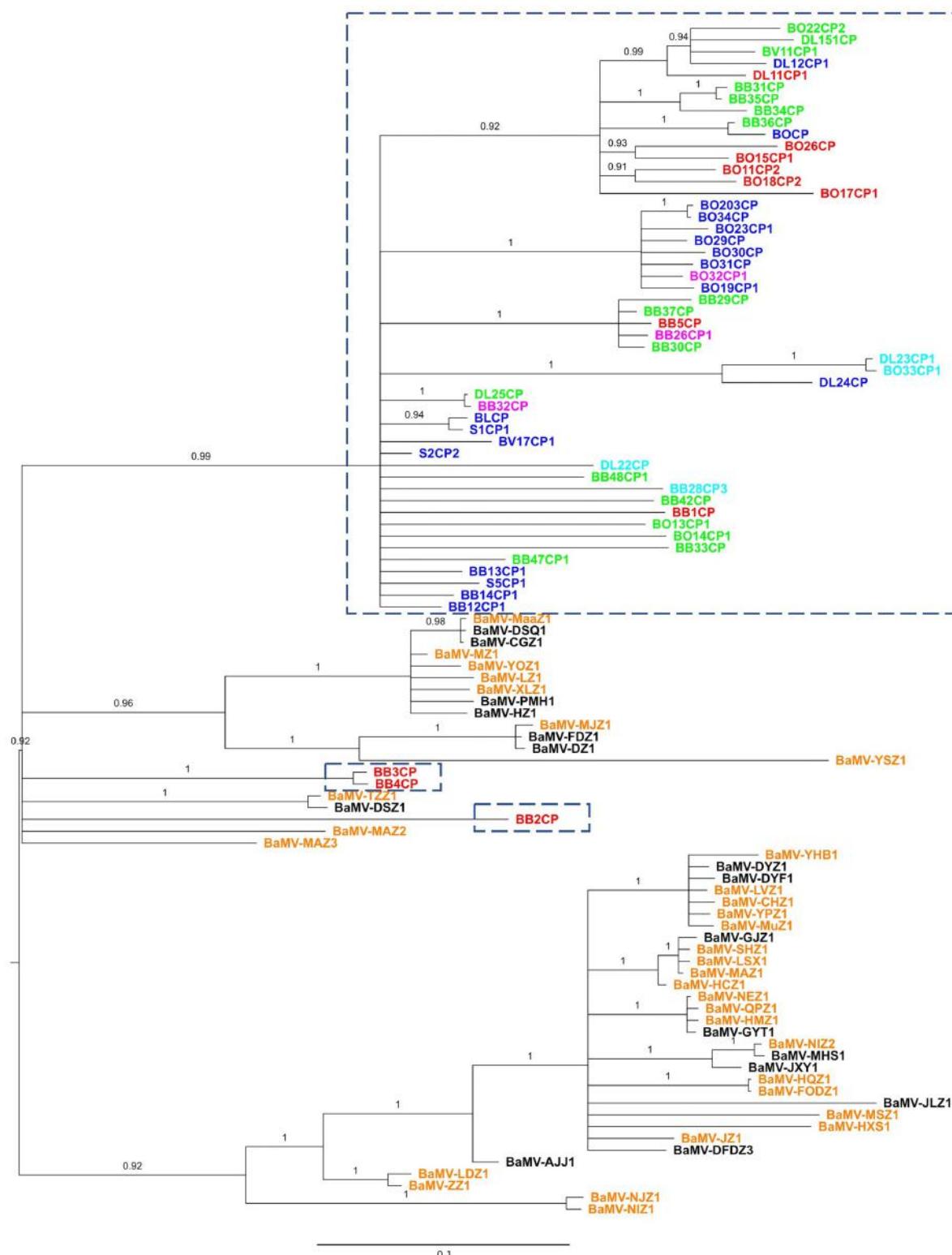
### Phylogeography of BaMV

Although BaMV has been detected in various parts of the world (Nelson and Borth, 2011), almost all available BaMV sequences are derived from samples obtained in

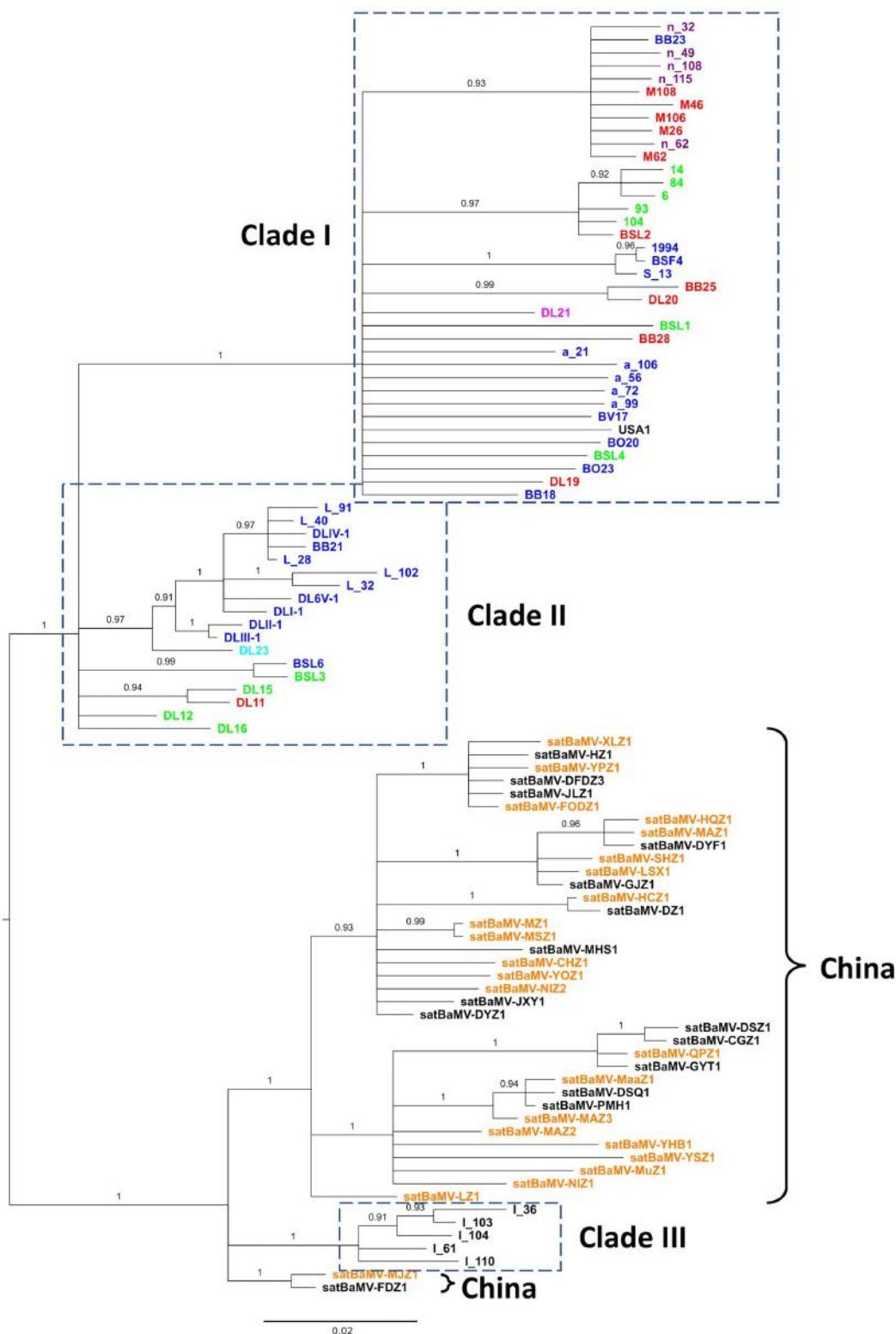
Taiwan. Recent additions from China (Lin et al., 2015, 2016) provide an opportunity to explore the evolutionary history among the BaMV isolates over a larger geographic area.

A total of 101 BaMV sequences, 53 from Taiwan and 48 from China, were used to reconstruct the phylogenetic relationship. The 528-nucleotide-long sequences (nucleotides 5611–6138, with BaMV-S genome as the reference, see Materials and Methods) encompass part of the CP gene, encoding 176 of the full-length 242 amino acid residues. To establish the character polarity, e.g., place of origin, a rooted tree is required. Although there are several approaches to rooting a phylogenetic tree (Kinene et al., 2016), the most commonly used are outgroup rooting and midpoint rooting. The outgroup rooting requires a homologous sequence known *a priori*. Typically, if possible, the outgroup rooting is preferred. Alternatively, the midpoint rooting is commonly used when no suitable outgroup is available. The midpoint rooting is found to be relatively successful at identifying the root of a phylogenetic tree (Hess and Russo, 2007). In this study, we used the BI and the ML methods to infer evolutionary histories among the BaMV isolates. For each method, we also reconstructed the trees with or without the presence of an outgroup sequence to evaluate the proper placement of the root. Of the two reconstruction methods, the BI method with midpoint rooting was able to produce a reasonably resolved phylogenetic relationship with high branch supports (**Figure 1**). On the other hand, the ML method produced a star phylogeny (polytomy) encompassing the majority of the sequences, thus rendering most of the relationships unresolved (see **Supplementary Material S3**). Furthermore, the phylogenetic relationships are better resolved with midpoint rooting than with outgroup rooting. This may be due to the fact that, over the region analyzed, the outgroup FoMV CP sequence shows a relatively low sequence identity of 29.5% with that of the BaMV CP. At the nucleotide level, the average number of nucleotide difference between the FoMV CP and BaMV CP gene is 276, while the average nucleotide difference between two BaMV sequences is only 64. It is likely that some nucleotide differences among the BaMV CP sequences are seen as “noises,” thus not contributing to the resolution of their relationships (Li and Graur, 1991; Mount, 2004). Nevertheless, despite the seemingly unresolved trees, the topologies of BI and ML trees are consistent in crucial aspects that are pertinent to our study. Therefore, we will focus our discussions based on the midpoint-rooted BI tree, as shown in **Figure 1**.

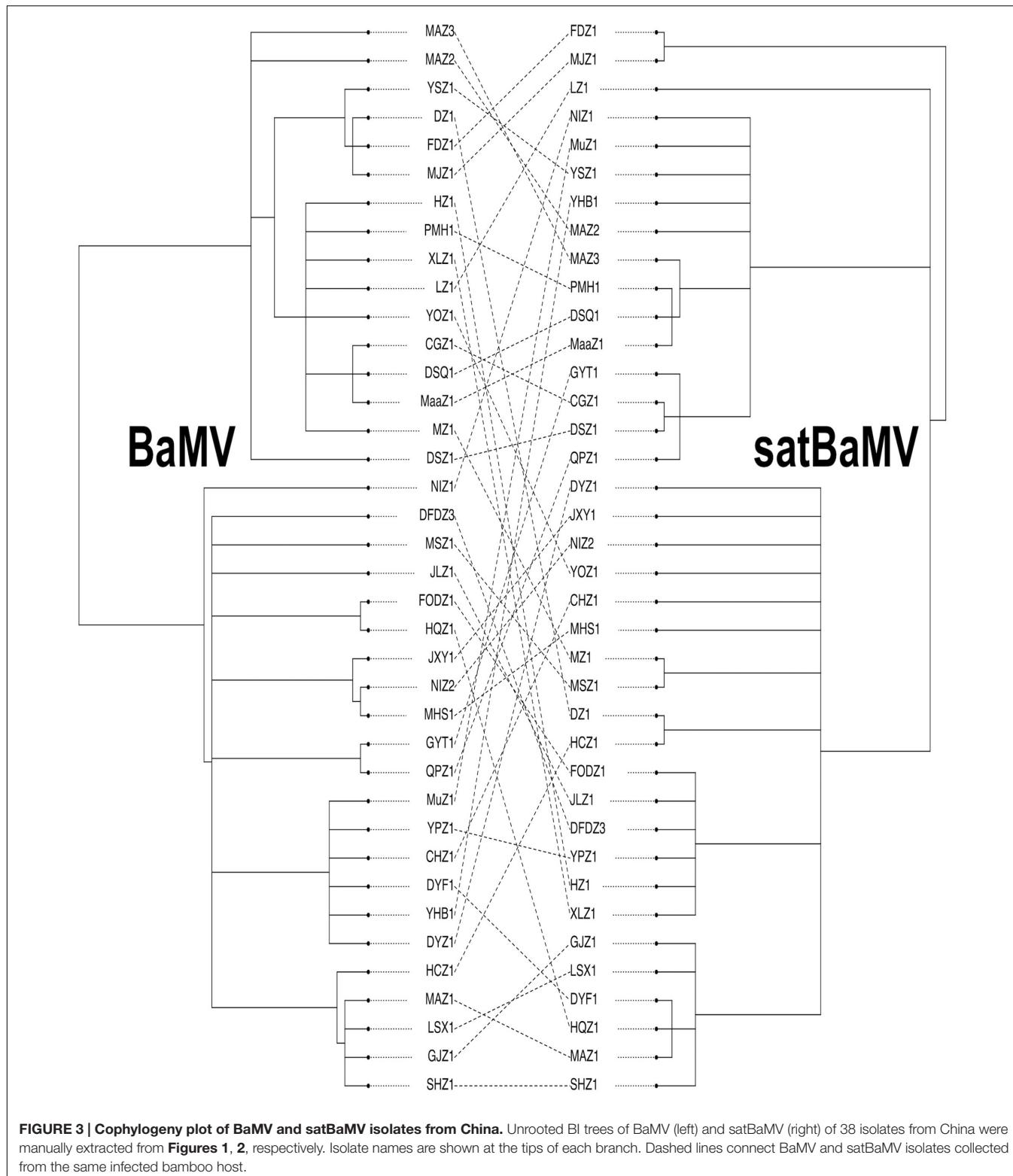
Several interesting patterns emerge from **Figure 1**. First, the majority of the isolates from Taiwan form a single clade and those from China two clades. There is no single instance in which the Chinese and Taiwanese isolates are found interspersed with each other. This complete coincidence between clade formation and geographic origin suggests that BaMV has a relatively independent evolutionary history in these two geographically proximate locations. Second, one of the Chinese clades forms the basal group of the tree, suggesting that the Taiwanese isolates originated in China. More limited studies, but with full-length genomic sequences, showed the



**FIGURE 1 | Phylogeny of BaMV isolates from Taiwan and China.** Partial sequences of the CP gene were aligned and phylogeny reconstructed with the BI method. Isolates within the dash-lined boxes are from Taiwan; the rest, with the prefix "BaMV-", are from China. Colored fonts indicate the general regions from which the isolates were collected: northern (blue), central (green), southern (red), northeastern (magenta), and eastern (cyan) Taiwan; southern (orange) and central (black) China. For location details, please see the **Supplementary Material S1**. The phylogeny is midpoint rooted. Numbers show the posterior probabilities (PPs). Branches with PP <0.9 are collapsed to polytomy.



**FIGURE 2 | Phylogeny of satBaMV.** Partial sequences of satBaMV isolates were aligned and phylogeny reconstructed with the BI method. Membership in previously identified three clades (Wang et al., 2014) are shown in the dash-lined boxes. Isolates from China are indicated with the right curly bracket symbols. Colored fonts, as shown in Figure 1, indicate the general regions from which the isolates were collected. It is to be noted that the isolates with the prefix “n-” in Clade I are sequences from the Hainan Island of China in our previous study. The phylogeny is midpoint-rooted. Numbers show the PPs. Branches with PP < 0.9 are collapsed to polytomy.



**FIGURE 3 | Cophylogeny plot of BaMV and satBaMV isolates from China.** Unrooted BI trees of BaMV (left) and satBaMV (right) of 38 isolates from China were manually extracted from Figures 1, 2, respectively. Isolate names are shown at the tips of each branch. Dashed lines connect BaMV and satBaMV isolates collected from the same infected bamboo host.

same pattern as well (Lin et al., 2016, 2017b). Third, multiple instances of independent BaMV introduction from China to Taiwan are discernible from the reconstructed phylogenetic trees.

While illuminating, these patterns are not unexpected, for the inferred direction of BaMV introduction followed the same typical route of species introduction/invasion from the mainland to an island (MacArthur and Wilson, 1967;

Lomolino et al., 2010). With the frequent exchange of goods and movement of both people and agricultural practices between Taiwan and China since ancient times, multiple, independent introductions of pathogens, including viruses, should be expected. However, we note that these BaMV isolates are not contemporaneous. The isolates from Taiwan were collected between 1994 and 2000 (Yeh et al., 2004) and those from China in 2014 (see **Supplementary Material S1** for details). It is not clear whether almost two decades of difference in evolution could drastically alter the inferred tree topology, thus resulting in an erroneous inference of movement direction (e.g., the current isolates from China may actually be derived from the older isolates from Taiwan). Nevertheless, our current analysis provides motivation for the need of a more systematic and wider geographic sampling of BaMV in the field.

## Phylogeography of satBaMV

Our previous analysis on the evolution of satBaMV revealed three well-supported clades. Clade I is composed of isolates from Taiwan (collected from various bamboo species from various locations on the island, see **Supplementary Material S1** for details) and southern China (Hainan Island, specifically). Clade II is composed of isolates from the infected Ma bamboo (*D. latiflorus* Munro) in Taiwan, and Clade III of isolates from India (Wang et al., 2014). The recently available satBaMV sequences from China are collected from a much wider geographic area (Lin et al., 2015, 2017a), thus providing an opportunity to infer the phylogeography of satBaMV isolates. In this study, a total of 98 sequences were analyzed, with 60 previously analyzed and published data and 38 recently added sequences from China. The 472-nucleotide-long sequences [nucleotides 271–742, with satBaMV-BSF4 genome (AY205227) (Lin and Hsu, 1994) as the reference] encompass part of the *P20* gene, encoding 146 of the full-length 183 amino acid residues. Since the CP gene from SPMV (GenBank accession number NC\_003847) is the most closely related sequence to satBaMV's *P20* gene, it is used as an outgroup to root the satBaMV tree. Over the region analyzed, the average number of nucleotide difference between SPMV CP and satBaMV sequence is 159, while the average difference between two satBaMV sequences is 23. It is apparent that the SPMV CP gene is only distantly related to the satBaMV CP gene. For this reason, we focus on midpoint-rooted BI tree for our discussion.

As shown in **Figure 2**, two interesting patterns emerge. First, the inferred midpoint-root bisects the satBaMV phylogeny at the point where it separates the previously defined Clades I and II into one of the dichotomous branches and Clade III into the other. That is, the satBaMV isolates collected from Taiwan are more closely related to each other than those collected from infected *B. vulgaris* bamboos in India (Wang et al., 2014). The same tree topology is also shown in the midpoint-rooted ML tree (see the **Supplementary Material S3**). This pattern suggests a deep genetic differentiation of satBaMV isolates between China and Taiwan. The only

region where Chinese and Taiwanese isolates co-mingled is the Hainan Island of China. However, we note that the outgroup-rooted BI tree shows a different tree topology, with the root being placed within the Clade I, from which Clades II, III, and the Chinese isolates are derived (see the **Supplementary Material S3**). Such a tree topology would imply an intriguing Taiwan-origin hypothesis for the way satBaMV evolved and migrated within the Asian continent. Second, more interestingly, the isolates from China, instead of scattering throughout the satBaMV phylogeny, are clustered together and are most closely related to those from India. This pattern further accentuates our finding that geographic distance (long distance between India and China versus short distance between Taiwan and China) does not seem to be a major determinant of phylogenetic relatedness among these satBaMV isolates. The relative lack of geographic differentiation is also seen within the East Asian continent. All of Chinese isolates are from three locations: two of them (Fujian Agriculture and Forestry University and Fuzhou National Forest Park) are in the city Fuzhou, Fujian; the other is in the city Chengdu (Wangjiang Park), Sichuan. Despite a distance of approximately 1,500 km between these two cities, there is no discernible clustering of isolates based on locations. As mentioned previously, there is an overwhelming clustering of Taiwanese and Chinese isolates, despite a much shorter distance of approximately 250 km between Taipei and Fuzhou. Therefore, we hypothesize that the Taiwan Strait forms a physical barrier, thus greatly limiting gene flow between mainland China and Taiwan. However, we note that satBaMV sequences from the Hainan Island, China are clustered with those from Taiwan, despite large swaths of South China Sea in between these two islands.

Taken together with the results from BaMV, we conclude that, despite gene flow, as evidenced by several independent introductions of BaMV from China to Taiwan, both BaMV and satBaMV in Taiwan have diverged greatly from those on the Asian continent. Although we do not have BaMV sequences from India or the Hainan Island, we predict that the Indian BaMV should be found allied with those in China, while the Hainan Island BaMV should cluster with those from Taiwan. Our current study also highlights the need for a more systematic sampling in various parts of the Asian continent proper and outlying islands, especially islands of Southeast Asia, to give us a more comprehensive picture on the origin, evolution, and phylogeography of BaMV and satBaMV.

## Coevolution between BaMV and satBaMV

When a parasite depends completely on its host for vital functions, it is ordinarily anticipated that both the parasite and host will coevolve. Therefore, speciation of the host should lead to corresponding speciation of the parasite, essentially coevolution via descent. Alternatively, evolution of parasite host range can sometimes lead to colonization of a new host species phylogenetically distant from the original host, thus

resulting in coevolution through colonization. The pattern of cophylogeny between the host species and the parasite species can be used to differentiate these two alternatives. Strict congruence between the host and parasite phylogenies suggests coevolution by descent. Otherwise, incongruence indicates events of shifted or expanded host range in the parasite. We reasoned that the cophylogeny study, commonly applied to investigating coevolution between host and parasite at the species level, can be used to investigate coevolution between BaMV and satBaMV at the population level as well. Of the 48 available BaMV sequences from China, 38 have corresponding satBaMV sequences that are isolated from the same infected bamboo (Lin et al., 2015, 2016, 2017a). These sequence pairs provide us an opportunity to explore the level of coevolution between the host BaMV and the parasite satBaMV.

We employed a cophylogeny plot, as shown in **Figure 3**, to visualize the degree of congruence between the BaMV and satBaMV phylogenies. **Figure 3** reveals a complex association pattern, indicating instances of incongruence between the two phylogenies (in the form of crisscrossing lines connecting individual BaMV/satBaMV pairs isolated from the same infected bamboo). We then used more quantitative approaches to explore the cophylogeny between BaMV and satBaMV from China.

Two general approaches are frequently used for cophylogeny study: global-fit and event-based methods, each has their advantages and disadvantages (Desdevises, 2007; Filippiak et al., 2016). In this study, we used *PACo* (Procrustean Approach to Cophylogeny) (Balbuena et al., 2013), a global-fit method, and *Jane* (Conow et al., 2010), an event-based method, to investigate the pattern of cophylogeny between BaMV and satBaMV sequences. An overall congruence would suggest that the BaMV/satBaMV pair in an infected host is frequently co-transmitted to a new host bamboo in their evolutionary history. In contrast, an incongruence between BaMV and satBaMV phylogenies would suggest relatively independent transmission histories of these two entities. Trees presented in **Figures 1, 2** are used as the data for each analysis.

*Procrustean Approach to Cophylogeny* uses the statistical process of Procrustes superimposition to obtain the Procrustes distance between two objects as a measure of “similarity in shape” (called “global goodness-of-fit,” symbolized by  $m^2_{XY}$ ). In the context of the cophylogeny analysis, the objects are the topologies of the host tree and the parasite tree. The significance of the observed  $m^2_{XY}$  can then be assessed by comparing to the distribution of sample  $m^2_{XY}$  generated through randomized associations between the host and parasite taxa. With the current topologies of the BaMV and satBaMV trees, the observed  $m^2_{XY}$  is 1.715, while the mean  $m^2_{XY}$  for the random samples is 2.047 (obtained from 100,000 randomizations). Since the resulting probability is  $1.71 \times 10^{-3}$ , we can reject the null hypothesis that the topology of the BaMV (host) tree cannot predict the topology of the satBaMV (parasite) tree. That is, at least a significant portion of the satBaMV tree topology depends on (i.e., can be predicted by) the BaMV

tree topology. Such a dependency suggests the presence of coevolution.

The advantage of the global-fit method, such as *PACo*, is that we can quickly obtain a statistical fit between two phylogenies without intensive computation. Its disadvantage is that we cannot have a detailed sense of what may have been the evolutionary events responsible for the observed pattern. For the event-based method of *Jane* (Conow et al., 2010), five mutually exclusive and exhaustive event types – cospeciation, duplication, duplication and host switch, loss, and failure to diverge – are assumed responsible for any given cophylogeny pattern. Various costs, or ranges of costs, are assigned to each event *a priori*. In the default setting, only the cospeciation event does not carry a cost, all the others have varying degrees of cost associated with them. Possible historical events (solutions) were found by minimizing the total costs. The minimum cost for the hypothesized historical event(s) can then be compared to a prescribed number of sample costs obtained by finding the minimum cost for each of the randomized association between the host and parasites. The details for the chosen parameters used in the analysis can be found in Section “Materials and Methods.” In the current cophylogeny study for BaMV and satBaMV, the best minimum cost for the current trees is 48, while the sample costs from 100 randomized associations ranged from 48 to 62, with a mean of 55.65 and a standard deviation of 3.15. This result showed that the observed BaMV and satBaMV topologies have a significantly lower cost than two randomly generated trees ( $p = 3.19 \times 10^{-8}$ ), suggesting that cospeciation is the major cause for the observed tree topologies. That is, BaMV and satBaMV isolates in general coevolve within the same infected bamboo individual.

Besides giving an overall statistical test, events that best fit our current cophylogeny pattern were also identified by the *Jane* analysis. Of the five possible event types, 16 “cospeciation,” 21 “duplication and host switch,” and 6 “loss” events were hypothesized. That is, a total of 43 evolutionary steps are needed to render the BaMV and satBaMV trees congruent. How do we interpret these hypothetical events in the ecological context of BaMV/satBaMV? It is interesting to note that, unlike other potexviruses, it is somewhat difficult to infect bamboos with BaMV/satBaMV preparation, even in the laboratory setting. Since bamboos are perennials, with some species having a lifespan of up to several decades, the difficulty in BaMV/satBaMV transmission would suggest a state of chronic infection. That is, the within-host population dynamics of viral infection is likely dominated by a set of BaMV/satBaMV that, given time, will coevolve from, say, the ancestral A/a sequences (or closely related mutant swarms) to the descendant A<sub>1</sub>/a<sub>1</sub>. If the ancestral set was also able to infect and establish in a different host successfully, the set can also coevolve to A<sub>2</sub>/a<sub>2</sub> via accumulation of phylogenetically informative mutations either independently, like genetic drift, or driven by natural selection, e.g., in the form of evolutionary arms race. That is, the typical ecological process of infection, in the context of BaMV/satBaMV biology, can be seen as the source of “cospeciation” event, as defined by the *Jane* analysis. If the

A/a set infects a bamboo that is already infected with a phylogenetically more distant coevolving set, say C/c, the A/a set can completely take over the C/c sequences, thus resulting in a cospeciation event. Or the process of superinfection can result in the successful establishment of A/c or C/a pairing, which, given time, will coevolve to A<sub>3</sub>/c<sub>3</sub> or C<sub>3</sub>/a<sub>3</sub>, respectively. Either way, a “loss” and a “duplication and host switch” event will then be counted in the *Jane* analysis.

Despite the apparent difficulty in transmitting BaMV/satBaMV from individual to individual bamboo, our cophylogeny analyses showed that successful establishment of infection or differential survival of superinfection by phylogenetically distinct BaMV and/or satBaMV sequences may not be uncommon. In fact, an *in planta* experiment showed differential accumulations of viral progeny between two BaMV isolates, suggesting one isolate having a fitness advantage over the other during coinfection, and presumably superinfection as well (Lin et al., 2017b). However, it is to be noted that all the Chinese isolate used in the cophylogeny analysis were collected in three areas and from many different bamboo species. All the sampling sites are in a botanical garden setting (W. Lin, personal communication), therefore, all infected bamboo hosts are presumed to be in close proximity to each other. However, we did not observe obvious clustering of sequences based on location of sampling (see **Figure 2**), suggesting that at least some of the BaMV/satBaMV isolates were preexisting before being placed in the same botanical gardens. Unfortunately, the actual geographic origins and natural host species are not clear for these BaMV/satBaMV isolates. Again, for a more detailed phylogeographic and cophylogenetic analyses, a more systematic collection of BaMV and satBaMV isolates in the field and full genomic sequences are needed.

## AUTHOR CONTRIBUTIONS

I-NW performed analyses and wrote the draft of the manuscript. W-BY conducted sequencing and submitted sequence information to GenBank. N-SL provided reagents and materials. All three edited the manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fmicb.2017.00886/full#supplementary-material>

**SUPPLEMENTARY MATERIAL S1 | BaMV and satBaMV isolates information.**

**SUPPLEMENTARY MATERIAL S2 | Map of BaMV and satBaMV sampling locations.**

**SUPPLEMENTARY MATERIAL S3 | (1)** Bayesian BaMV CP gene tree with FoMV CP gene as the outgroup. Numbers show the posterior probabilities (PP). Branches with PP < 0.9 are collapsed to polytomy. **(2)** Maximum likelihood BaMV CP gene tree with midpoint rooting. Numbers show the bootstrap values (out of 100). Branches with bootstrap values < 375 (out of 500) are collapsed to polytomy. **(3)** Maximum likelihood BaMV CP gene tree with FoMV CP gene as the outgroup. Numbers show the bootstrap values (out of 500). Branches with bootstrap values < 375 are collapsed to polytomy. **(4)** Bayesian satBaMV tree with SPMV CP gene as the outgroup. Branches with posterior probability < 0.9 are collapsed to polytomy. **(5)** Maximum likelihood satBaMV tree with midpoint rooting. Branches with bootstrap values < 375 (out of 500) are collapsed to polytomy. **(6)** Maximum likelihood satBaMV tree with SPMV CP gene as the outgroup. Branches with bootstrap values < 375 (out of 500) are collapsed to polytomy.

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# Production of Japanese Encephalitis Virus Antigens in Plants Using Bamboo Mosaic Virus-Based Vector

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Japanese encephalitis virus (JEV) is among the major threats to public health in Asia. For disease control and prevention, the efficient production of safe and effective vaccines against JEV is in urgent need. In this study, we produced a plant-made JEV vaccine candidate using a chimeric virus particle (CVP) strategy based on bamboo mosaic virus (BaMV) for epitope presentation. The chimeric virus, designated BJ2A, was constructed by fusing JEV envelope protein domain III (EDIII) at the N-terminus of BaMV coat protein, with an insertion of the foot-and-mouth disease virus 2A peptide to facilitate the production of both unfused and epitope-presenting for efficient assembly of the CVP vaccine candidate. The strategy allowed stable maintenance of the fusion construct over long-term serial passages in plants. Immuno-electron microscopy examination and immunization assays revealed that BJ2A is able to present the EDIII epitope on the surface of the CVPs, which stimulated effective neutralizing antibodies against JEV infection in mice. This study demonstrates the efficient production of an effective CVP vaccine candidate against JEV in plants by the BaMV-based epitope presentation system.

**Keywords:** bamboo mosaic virus-based vector, chimeric virus particles (CVPs), foot-and-mouth disease virus 2A, Japanese encephalitis virus, vaccine, plant-made

## INTRODUCTION

Japanese encephalitis virus (JEV), the causal agent of Japanese encephalitis (JE), is a plus-strand RNA virus of the family *Flaviviridae* (Vaughn and Hoke, 1992; Unni et al., 2011). JE is a major public health problem in Asia, causes up to 50,000 encephalitis cases and 10,000 deaths annually in humans (Campbell et al., 2011; Unni et al., 2011; Li et al., 2014; Tarantola et al., 2014; Cappelle et al., 2016). With the lack of specific antiviral treatment, vaccination against JEV is crucial for prevention (Li et al., 2014), and is recommended by the World Health Organization (WHO) for the at-risk populations (WHO, 2015). However, the successful implementation of vaccination programs in such areas may depend largely on the cost-effectiveness and safety concerns of the vaccines, similar to the cases for a close relative of JEV, the West Nile virus (Zohrabian et al., 2006; Martina et al., 2010; Chen, 2015).

Currently inactivated JEV vaccines prepared from infected mouse brains (BIKEN or JEVAX) or primary hamster kidney cells and a live attenuated vaccine (SA14-14-2) have been successfully developed to control JEV infection (Mackenzie et al., 2004; Ghosh and Basu, 2009). Nevertheless, the use of inactivated JEV vaccine does not confer sufficient long-term immunity to provide

effective protection (Mackenzie et al., 2004; Ghosh and Basu, 2009). In addition, there are also concerns of side effects (Shlim and Solomon, 2002). Accordingly, WHO has designated JEV vaccines as a high-priority target for development of a new vaccine to fight against JE worldwide (Tsai, 2000).

The applications of plants as bioreactors to produce valuable proteins, including vaccines, have attracted considerable interests in recent years (Takeyama et al., 2015). Plants can produce large volumes of products efficiently and can have significant advantages in decreasing manufacturing costs (Thomas et al., 2011; Moustafa et al., 2016). The production of foreign proteins can be achieved through stable transformation of the nuclear or chloroplast genomes, or the transient expression mediated by *Agrobacterium*- or virus-based vector systems (Lico et al., 2008; Chen and Lai, 2013). Among these commonly used approaches, virus-based transient expression vector systems are particularly promising for rapid expression of recombinant proteins at levels higher than with stable transgenic plants (Daniell et al., 2009).

Plant viral vector systems explore various strategies for recombinant protein expression, including gene insertion or substitution, modular or deconstructed vector design, and protein fusion (peptide display) (Lico et al., 2008). The presentation of heterologous epitopes on plant virus particles is very convenient for peptide-based production of therapeutics and vaccines. The protein fusion strategy has been used extensively to display target peptides on the surface of chimeric virus particles (CVPs) to enhance immunogenicity (e.g., Gerloni et al., 2000; Smith et al., 2006; Massa et al., 2008; Hassani-Mehraban et al., 2015), and to facilitate easy antigen purification. In a previous study, we have reported the use of a bamboo mosaic virus (BaMV)-based vector as an effective epitope presentation system, and demonstrated that the foot-and-mouth disease virus (FMDV) VP1 epitopes expressed on BaMV CVPs can effectively induce humoral and cell-mediated immune responses in swine and provide full protection against FMDV challenges in that host (Yang et al., 2007). This BaMV-based CVP vector system presents an alternative approach for the development of a vaccine candidate against JEV.

Japanese encephalitis virus RNA contains a single open reading frame (ORF) that codes for a polyprotein which is proteolytically processed into three structural proteins designated envelope (E), membrane (M), and capsid (C) and seven non-structural proteins, NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 (Unni et al., 2011). The E protein appears to play an important role in viral attachment, membrane fusion for entry into the host cell (Stiasny and Heinz, 2006), virus assembly and maturation, and most notably, inducing virus-neutralizing antibodies (Mason et al., 1989; Kurane, 2002). The key domain of E protein, EDIII, forms a  $\beta$ -barrel type structure resembling the immunoglobulin constant domain and can be independently folded as an individual fragment by forming a disulfide bond (between residues 304 and 335) to maintain its conformation (Wu K. P. et al., 2003). Moreover, neutralizing epitopes in the EDIII have been identified on the lateral surface (Cecilia and Gould, 1991; Seif et al., 1995; Lin and Wu, 2003). Therefore, EDIII represents a potential antigen for producing vaccine candidates.

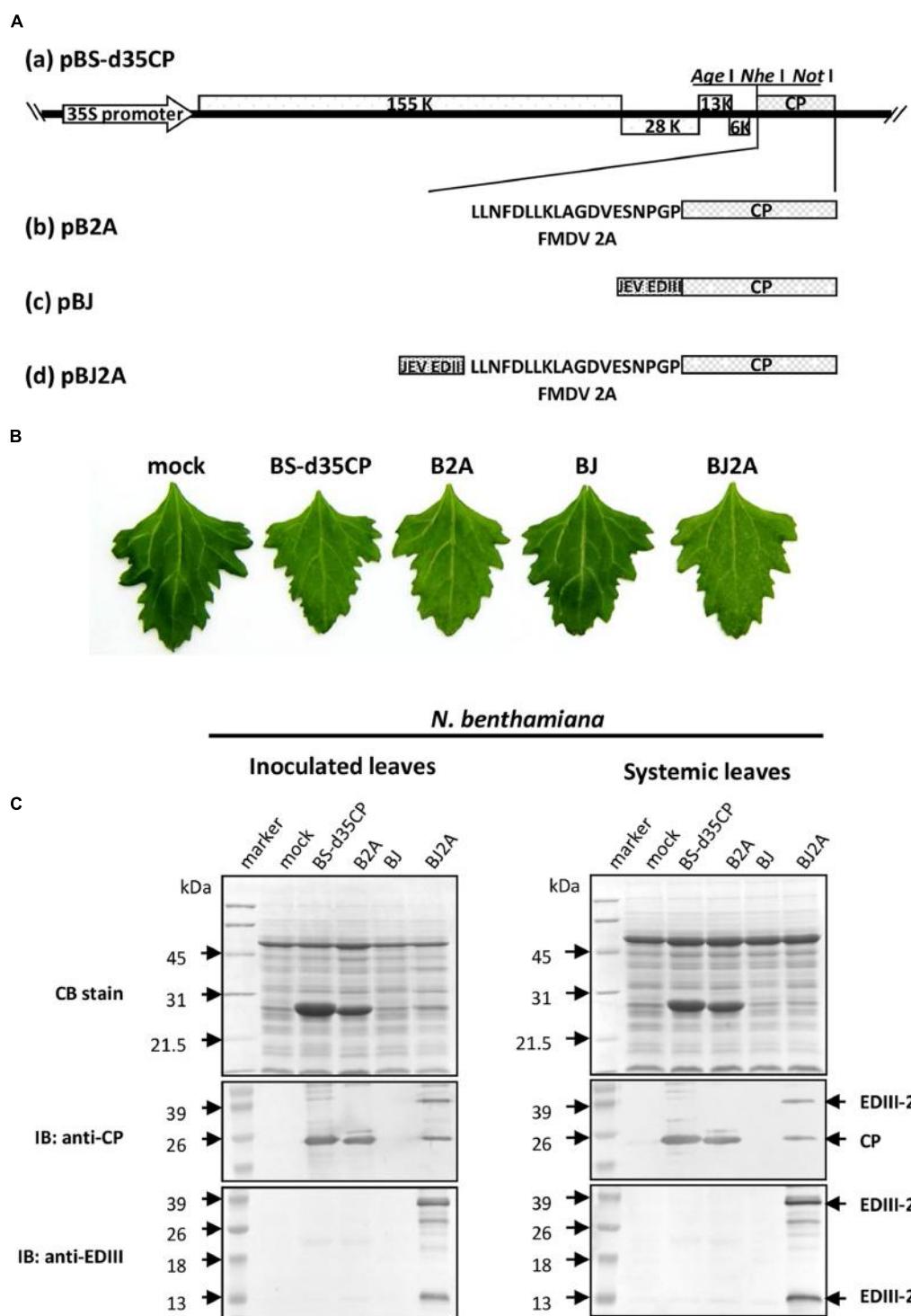
The use of autonomously replicating viruses as expression vectors provides an attractive means for the transient expression of CVPs displaying JEV EDIII antigens in plants. However, the sizes of the epitope presented in our previous BaMV-based CVP vector was limited to 37 amino acids (Yang et al., 2007), which is also a common barrier encountered by other CVP-based expression systems (e.g., Bendahmane et al., 1999; Jiang et al., 2006; Uhde-Holzem et al., 2010; Zhang et al., 2010). For epitopes with larger sizes, such as the EDIII epitope of JEV, or other unfavorable structural features, alternative strategies are required to improve the survival rate and the stability of the fusion proteins.

In this study, we aimed to develop a BaMV-based CVP vaccine against JEV by fusing JEV EDIII to BaMV coat protein (CP) and displaying the EDIII epitopes on the surfaces of CVPs. To overcome the size-limitations of the epitope-presentation systems, we have adopted the strategy of Cruz et al. (1996) by inserting the 2A co-translational dissociation sequence from FMDV (designated 2A) to the junction of JEV EDIII and BaMV CP, providing enhanced solidity of the CVPs while retaining the presentation of EDIII epitopes on portions of virion surfaces. Detailed analysis were performed to investigate the genetic stabilities of the chimeric virus and the proportions of EDIII-2A-BaMV CP fusion proteins assembled into CVPs among those produced in plant cells. Immunization assays were also conducted to examine the effectiveness of these chimeric CVPs to stimulate the immune responses in mice. Evidence was provided to support that the BaMV-based CVP may offer an alternative vaccine candidate to elicit the generation of neutralization antibodies in mice.

## MATERIALS AND METHODS

### Construction of Chimeric BaMV Infectious Clone

The infectious recombinant constructs used in this study were derived from a mutant BaMV cDNA plasmid, pBS-d35CP (Yang et al., 2007) (**Figure 1A**), in which the N-terminal 35 amino acids of CP have been deleted. The coding sequence of JEV (CH2195LA strain) EDIII region, from nucleotide position 874 to 1206, was amplified with primers 5' *ggactagttaccatggacaaactggccctgaaaggc* 3' and 5' *cgttccagtcgcagacattgcggccgcgtgttccgttttg* 3' (with JEV EDIII coding sequences italicized, and restriction sites for *SpeI*, and *NotI*, respectively, underlined) by PCR using plasmid pET32a/LD3 (Wu S. C. et al., 2003) as the template. The PCR-amplified fragment was purified and inserted into plasmid pBS-d35CP at the *NheI* and *NotI* site, resulting in plasmid pBJ (**Figure 1A**). The DNA fragment coding for the FMDV 2A peptide (LLNF DLLKLAGDVESNPGP) (Ryan et al., 1991) was amplified by PCR with primers 5' *ggcttagcgccgcgcgtgtgaatttgaccccttaagcttgcgg* 3' and 5' *cctggcccgggtccgggttgactcgacgtcccccaagcttaagaagg* 3' (with FMDV 2A coding sequence italicized, restriction sites underlined for *NheI*, *NotI*, in conjunction, and *PspOMI*, respectively, and complementary sequences in boldface). Plasmid pB2A was constructed by inserting the FMDV 2A coding sequence at the



**FIGURE 1 |** Japanese encephalitis virus (JEV) EDIII is expressed in plants infected with chimeric BaMV. **(A)** Schematic representation of the recombinant constructs based on BaMV genome. **(B)** Infectivity and symptom of various recombinant BaMV construct on *Chenopodium quinoa*. Leaves inoculated with H<sub>2</sub>O (mock) or recombinant plasmids pBS-d35CP, pB2A, pBJ, or pBJ2A were shown. The photos were taken at 10 days post-inoculation (dpi). **(C)** SDS-PAGE separation and immunoblot analysis of proteins extracted from inoculated or systemically infected leaves of *N. benthamiana*, as indicated on top of each panel. Leaves were H<sub>2</sub>O-inoculated (mock) or inoculated with recombinant plasmids pBS-d35CP, pB2A, pBJ, or pBJ2A as indicated. Total proteins extracted from inoculated leaves (accounting for 1 mg fresh weight of leaf) from each treatment were separated in a 12% SDS-PAGE (Top panel), and stained with coomassie blue (CB). The proteins were transferred to PVDF membranes and reacted with antisera against BaMV CP (anti-CP, middle panel), or JEV EDIII (anti-EDIII, bottom panel), respectively. The relative molecular weights (in kDa) are given on the left of each panel, and positions of each target proteins on the right. IB, immuno-blot.

5'-terminus of the truncated CP ORF of pBS-d35CP with proper restriction enzyme digestions (**Figure 1A**). The above-mentioned JEV EDIII coding sequence was inserted into plasmid pB2A at the *NheI* and *NotI* site to give plasmid pBJ2A (**Figure 1A**). The identities of all plasmids were confirmed by nucleotide sequencing.

### Preparation of Recombinant EDIII (rEDIII)

Japanese encephalitis virus EDIII fragments were obtained from pET32a/LD3 plasmid (Wu S. C. et al., 2003) by digestion with *NcoI* and *NotI*, and cloned into plasmid pET21d (Novagen) at the respective sites for over-expression in *Escherichia coli*. Methods used for expression and purification of rEDIII protein were as previously reported (Seif et al., 1995), except that the *E. coli* strain BL21(DE3) (Novagen) was transformed with the rEDIII-expression plasmid and grown overnight in LB medium in the presence of ampicillin (50 µg ml<sup>-1</sup>). The cells were then diluted 50-fold in LB medium containing ampicillin and grown at 37°C. The rEDIII protein was further dialyzed against phosphate-buffered saline (PBS). The purified rEDIII was further subjected to raise specific antiserum in rabbits following standard procedures (Lin and Chen, 1991).

### Protein Analysis of the Infected Plant Tissue and Stability of Chimeras during Sequential Transmission

The genetic stability of BJ2A chimeric virus was tested using local-lesion host *Chenopodium quinoa*, while the systemic movement of the chimeric virus was tested on systemic-infection host *Nicotiana benthamiana*. The infectious viral cDNA clones of pBS-d35CP, pB2A, pBJ, and pBJ2A were inoculated onto *N. benthamiana* or *C. quinoa* as previously reported (Yang et al., 2007). The plants were grown in a greenhouse exposed to normal daylight. After local lesions appeared on the pBJ2A-inoculated leaves of *C. quinoa* at 10 days post-inoculation (dpi), leaves were excised and ground in deionized H<sub>2</sub>O (1:10; weight:volume). The crude sap was mechanically inoculated to healthy *C. quinoa*. The above-mentioned procedure was repeated for nine times, and the progeny virus, BJ2A, on *C. quinoa* leaves was assayed each time to examine the stability of the chimeric virus during successive passages in plants. Total proteins extracted from inoculated leaves were separated by electrophoresis on a 12% polyacrylamide gel containing 1% sodium dodecyl sulfate (SDS-PAGE), and stained with coomassie blue (CB). The proteins were then transferred to PVDF membranes (Millipore) and reacted with antisera against BaMV CP (Lin and Chen, 1991) or rEDIII, respectively.

### Detection of EDIII in Inoculated Plants by Enzyme-Linked Immunosorbent Assay (ELISA)

The plant-made JEV EDIII proteins in *C. quinoa* leaves inoculated with pBJ2A were examined by indirect ELISA using the rabbit antiserum against rEDIII. ELISA was performed as described previously with minor modifications (Saejung et al., 2007). The bound protein-antibodies were detected with biotin-conjugated goat anti-rabbit IgG using the

VECTASTAIN Elite ABC kit (avidin biotinylated peroxidase; Vector Laboratories). Following color development, the absorbance at 450 nm was measured on an ELISA reader (Spectramax M2, Molecular Device, USA). Known amounts of purified JEV rEDIII protein was used to establish the standard curve for quantification. Protein extract from a healthy plant was used as a negative control.

### BJ2A CVP Purification

*Chenopodium quinoa* was chosen as the host plant for the production of BJ2A CVPs to avoid the potential side effects of nicotine and other alkaloids present in *N. benthamiana* (Mishra et al., 2015). Leaves of *C. quinoa* inoculated with pBJ2A were harvested at 10 dpi. The BJ2A CVPs were subsequently purified from the leaves and the yield was determined spectrophotometrically by absorbance at 280 nm as described previously (Lin and Chen, 1991). Purified BJ2A CVPs were dissolved in BE buffer (50 mM Borate, pH 8.0, 1 mM EDTA), then stored at -20°C until used. Chimeric BJ2A virions were separated on a 12% SDS-PAGE. The protein bands corresponding to the EDIII-2A-CP fusion protein or cleaved CP on the gel were quantitated by using the Alpha Imager 2200 V5.04 documentation and analysis system.

### Immunoelectron Microscopy

Methods used for the examination of chimeric BJ2A virions by immunoelectron microscopy were as previously reported (Lin, 1984). Gold-labeled antibodies specific for BaMV CP (Lin and Chen, 1991), JEV EDIII, and pre-immune serum were used in the respective experiments. The grids were finally negatively stained with 2% uranyl acetate and examined with transmission electron microscopy (Philips CM 100 Bio) at 80 KV. Control grids were treated with pre-immune rabbit antiserum.

### Mouse Immune Response

Three groups of 6-week-old female BALB/c ByJ mice, six mice per group, obtained from the National Laboratory Animal Center (Taipei, Taiwan), were immunized by intraperitoneal injection. The care of the animals was provided in accordance with guidelines approved by the animal committee of the Institute of Biomedical Sciences, Academia Sinica. One group was immunized with 200 µg BJ2A CVPs. The second group was immunized with 30 µg rEDIII as the positive control. The third group was injected with saline as the negative control. All the mice were boosted with the same dose on day 12. The primary antigens were emulsified in Freund's complete adjuvant (Difco) and boosters emulsified in Freund's incomplete adjuvant (Sigma). Sera were collected on days 0 and 49. The titers and reactivity of sera were tested using indirect ELISA, indirect immunofluorescence assay and plaque reduction neutralization, described as follows.

### Analysis of EDIII-Specific Antibody in Mice Sera by ELISA

Serum samples were collected by periorbital route and heat-inactivated at 56°C for 30 min. JEV EDIII-specific antibodies

in serum samples were analyzed by indirect ELISA as described previously (Yang et al., 2007), except that ELISA plates (Nunc) were coated with rEDIII (1 µg per well) as antigens, and bound antibodies were detected with biotin-conjugated goat anti-mouse IgG (H+L). Following color development, the absorbance at 450 nm was measured on an ELISA reader. For background reactions, mice pre-immune sera were used in the ELISA.

### Indirect Immunofluorescence Assay

To analyze whether the BJ2A CVPs elicited the production of effective JEV EDIII-specific antibodies in the immunized mice, indirect immunofluorescence assay was performed as described previously (Wu et al., 2002), except that BHK-21 cells were infected with JEV (RP-9 strain) and the sera obtained from the immunized mice were pooled and 100-fold diluted. Fluorescence was observed with a Leica fluorescence microscope. Cell nuclei were visualized by 4, 6-diamidino-2'-phenylindole (DAPI) staining in 0.9% sodium chloride. Pictures were taken using an inverted fluorescent microscope (Leica) by double exposure of the same fields with filters for FITC and DAPI.

### Neutralization Test

Neutralizing antibody was assayed by plaque reduction neutralization test (PRNT) in BHK-21 cells as previously described (Chen et al., 2005) with minor modifications. Briefly, serum samples were subjected to a serial twofold dilution in 5% fetal bovine serum (FBS)-PBS on ice. Then, equal volumes of infectious JEV in minimum essential medium (MEM) supplemented with FBS were mixed with the serially diluted serum sample to make a mixture containing approximately 100 pfu of virus per well. The virus-antibody complex was added to six-well plates (in triplicates) containing confluent monolayers of BHK-21 cells. The plates were incubated at 37°C for 1 hr with gentle rocking every 15 min. The wells were then overlaid with 2 ml of 1% methyl cellulose prepared in MEM, supplemented with 5% FBS and incubated at 37°C in 5% CO<sub>2</sub> for 4 days. Plaques were stained with naphthol blue black and counted. The neutralizing antibody titer was calculated as the reciprocal of the highest dilution resulting in a 70% reduction of plaques compared to that of a control of virus without antibody added.

## RESULTS

### Production of JEV EDIII Using Chimeric BaMV Vectors in Plants

To achieve better yield and stability of JEV EDIII in plants, we explored two different strategies by using BaMV-based vector: (i) direct fusion of JEV EDIII to the N-terminus of truncated BaMV CP, and (ii) insertion of FMDV 2A co-translational dissociation peptide sequence in between JEV EDIII and BaMV CP. The first approach was expected to result in higher yield of the epitope, with JEV EDIII presented on every BaMV CP subunits, at the cost of losing virion stability. The second approach allowed for

the production of both the JEV EDIII-2A-BaMV CP recombinant protein and the unfused BaMV CP, leading to the display of JEV EDIII on only portions of the chimeric BaMV virions, with the expected increase in stability of the CVP. Accordingly, two recombinant plasmids, pBJ and pBJ2A, were constructed based on a modified BaMV vector pBS-d35CP (**Figure 1A**). The infectivity of the recombinant viral vectors was assayed in both *N. benthamiana* and *C. quinoa*. The result revealed that infection with pBJ2A led to stronger mosaic symptoms than that with pBS-d35CP in *N. benthamiana*, whereas chlorotic local lesions distinct from those caused by pBS-d35CP were observed after pBJ2A inoculation in *C. quinoa* (**Figure 1B**). In contrast, inoculation with pBJ did not cause any visible symptom on both *N. benthamiana* and *C. quinoa* (**Figure 1B**).

To determine whether fusion proteins were produced properly in plants inoculated with the chimeric viruses, total proteins from the inoculated leaves of *N. benthamiana* infected with distilled water (mock), pBS-d35CP, pB2A, pBJ, or pBJ2A were subjected to analyses by SDS-PAGE (**Figure 1C**) and western blotting assay using BaMV CP-specific antibodies (**Figure 1C**, middle panel). As anticipated, no BaMV CP was detected in protein extract of mock-inoculated leaves (**Figure 1C**, mock). The FMDV 2A-BaMV CP fusion protein and N-terminal 35-amino-acid truncated BaMV CP (**Figure 1C**, middle panel, B2A) were both detected in the protein extract of pB2A-inoculated leaves, which migrated slightly faster than the chimeric CP from pBJ2A-inoculated leaves (**Figure 1C**, middle panel, BJ2A). In contrast, no BaMV CP was detected in the pBJ-inoculated leaves (**Figure 1C**, middle panel, BJ). To further verify that chimeric CP generated from pBJ2A-inoculated leaves harbored JEV EDIII peptide, western blotting analysis using rEDIII-specific antiserum was performed (**Figure 1C**, lower panel). Two proteins of 36.8 and 14.1 kDa were detected by rEDIII-specific antiserum, corresponding to the chimeric EDIII-2A-CP fusion protein and the free JEV EDIII, respectively (**Figure 1C**, lower panel, BJ2A). In contrast, no protein band was detected by the rEDIII-specific antiserum in protein extracts from leaves inoculated with pBS-d35CP, pB2A, or pBJ (**Figure 1C**, lower panel). Similar results were obtained when total protein extracts from systemic leaves of the infected *N. benthamiana* were assayed by western blotting using either BaMV CP- or rEDIII-specific antisera, respectively (**Figure 1C**, right panel). The above results suggested that the incorporation of FMDV 2A peptide did not affect the replication and systemic movement of the chimeric viruses, and indeed improved the infectivity of pBJ2A as compared to pBJ in both host plants tested.

### Stability of BJ2A Chimeras during Successive Passages in *C. quinoa*

To examine the stability of the chimeric BJ2A virus during successive passages in plants, infectious recombinant pBJ2A plasmid was inoculated onto *C. quinoa* leaves to generate the initial inoculum, designated P0, which was then subjected to nine sequential transmissions (P1 through P9) in *C. quinoa*. The presence of the EDIII-2A-CP fusion protein was monitored at each transfer by western blot analysis using specific antisera. All

inoculated leaves developed lesions similar in appearance and number to those observed on P0-infected plants. Results from western blot analyses using antisera specific to BaMV CP or JEV EDIII clearly identified fusion-form BJ2A CP (36.8 kDa), EDIII2A polyprotein (14.1 kDa) and free CP (22.7 kDa) in total protein extracts from all the serially inoculated *C. quinoa* plants (**Figure 2**). After quantification of the proteins by using ELISA, the level of JEV EDIII expressed in the leaves of the *C. quinoa* plants was estimated to be  $8.9 \pm 4.3 \mu\text{g mg}^{-1}$ , corresponding to  $0.89 \pm 0.43\%$  of total soluble protein (TSP). The result demonstrated that the insertion of foreign coding sequences could be stably maintained in the genome of the chimeric virus over serial passages.

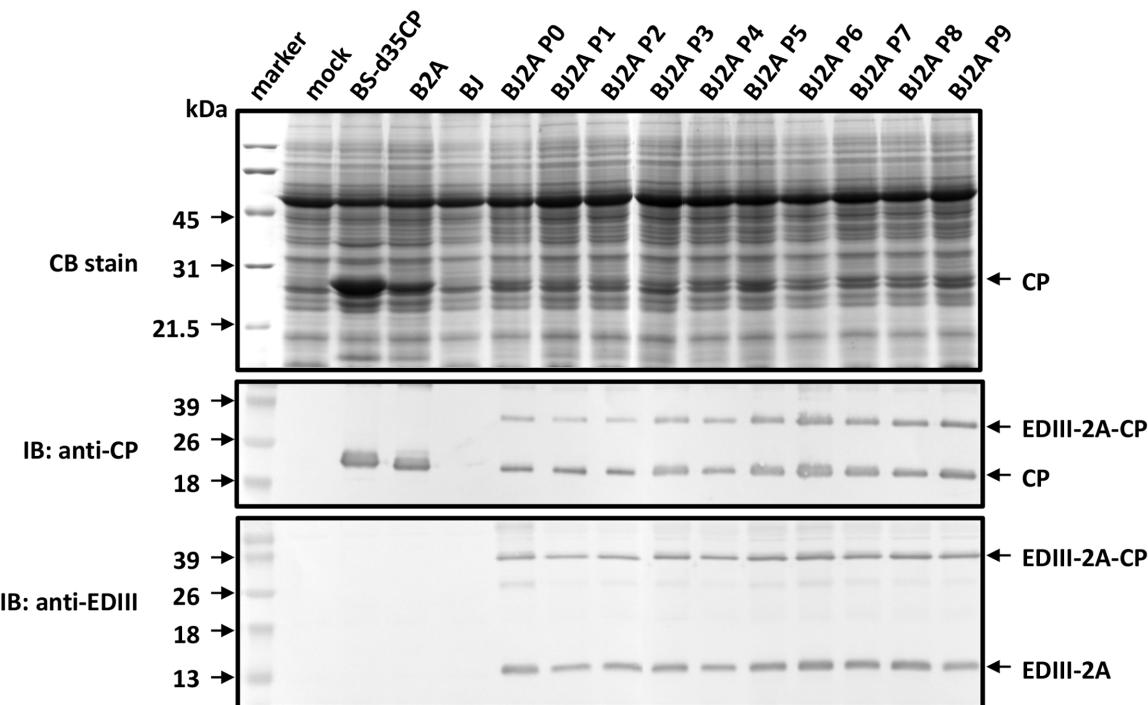
## JEV EDIII Peptides on the Outer Surfaces of CVPs

Following the successful observation of the stable expression of both BJ2A fusion proteins and free CP in the inoculated plants, it is important to examine whether the CVPs could be properly assembled with the EDIII peptides presented on the outer surfaces. Results of the electron microscopy observation revealed that the BJ2A CVPs appeared typically filamentous with lengths approximately the same as those of the wild type BaMV virions (480 nm) (**Figures 3A–C**). Immunogold labeling using polyclonal antibodies against EDIII confirmed that the foreign EDIII epitopes were accessible and exposed on the surface of

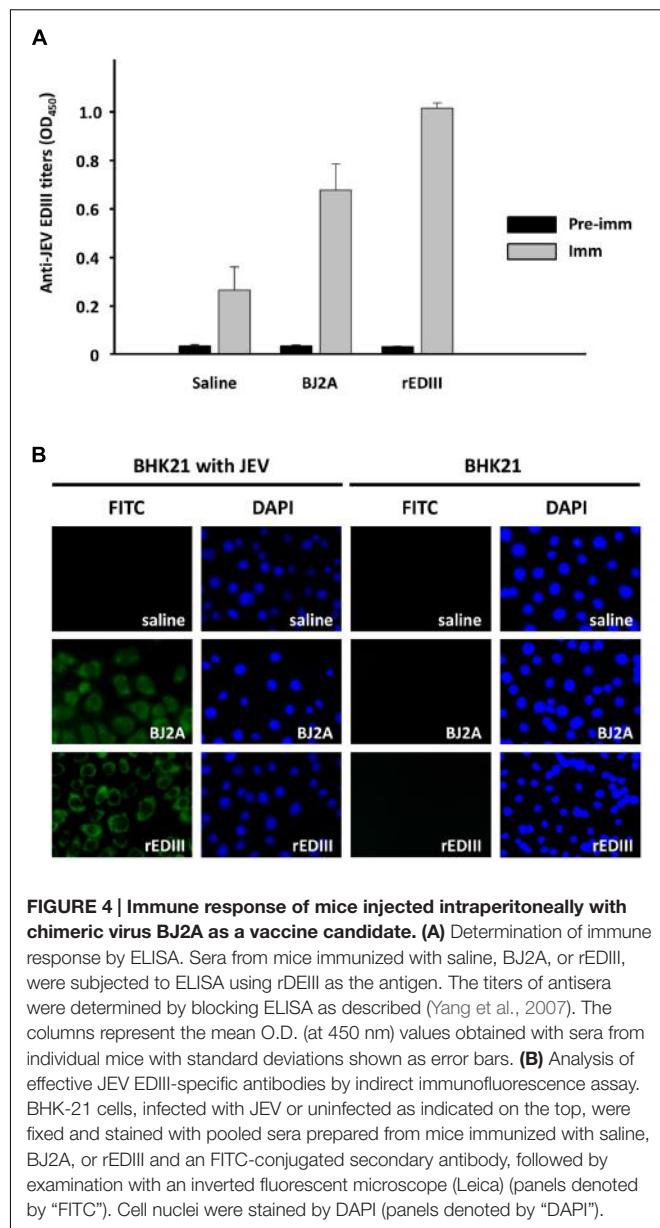
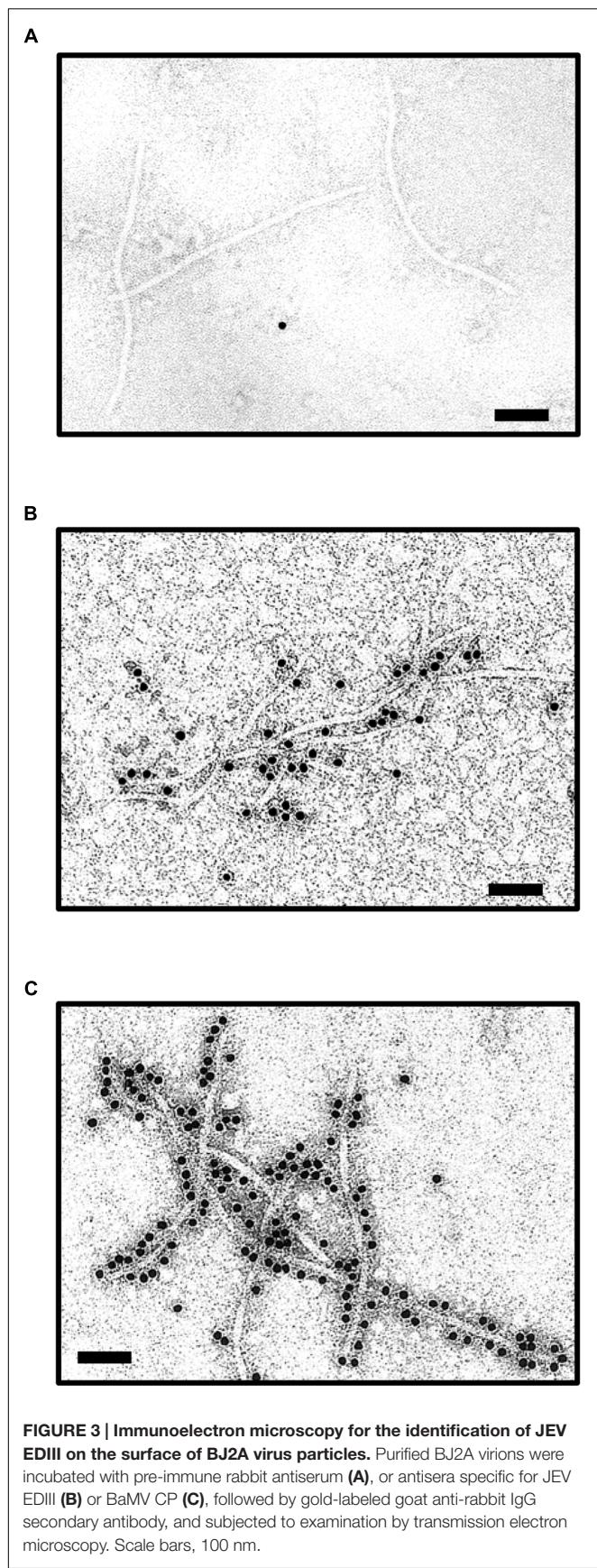
the CVPs (**Figure 3B**). As controls, BJ2A CVPs were labeled with gold-conjugated antiserum against BaMV CP (**Figure 3C**), but not with pre-immune serum (**Figure 3A**). The results demonstrated that the CPs and EDIII-2A-CP fusions can be properly assembled into BJ2A CVPs.

## Induction of Anti-JEV Antibody in Mice Immunized with Purified BJ2A CVPs

To determine immunogenicity and efficacy of the CVPs with target proteins presented on the surface, immune responses in mice were assayed as described in section “Materials and Methods.” Blood samples from each group were collected from the periorbital route at days 0 and 49 after immunization. The reactivity to JEV EDIII by sera from BJ2A CVPs-immunized mice was examined by ELISA. The result showed that BJ2A-immunization elicited high levels of anti-EDIII antibodies in sera of the treated mice, similar to those observed for sera from rEDIII-immunized mice as a positive control (**Figure 4A**). The antibody reactivity was weak in the negative control group, which received a combination of saline and adjuvant throughout the experiment (**Figure 4A**). Subsequently, the reactivity of BJ2A CVP-immunized sera was tested by immunofluorescence assay in JEV infected BHK 21 cells. The results showed that sera from BJ2A CVP- or rEDIII-immunized mice recognized the JEV infected BHK 21 cells (**Figure 4B**), demonstrating their *ex vivo* reactivity. As a negative control, no fluorescence was detected



**FIGURE 2 | Analysis of the stability of the chimeric BJ2A over serial passages in *C. quinoa* plants by SDS-PAGE and immunoblot.** Leaves were H<sub>2</sub>O-inoculated (mock) or inoculated with recombinant plasmids pBS-d35CP, pB2A, pBJ, or pBJ2A, respectively. BJ2A P0 denotes the initial inoculation with the plasmid DNA as inoculum, whereas P1 to P9 indicate the 1st to 9th passage using crude leaf sap from P0 as inocula, respectively. SDS-PAGE and immunoblot assays were performed as described in **Figure 1C**.



when using sera from the group that received a combination of saline and adjuvant throughout the experiment, nor in non-infected BHK 21 cells (**Figure 4B**).

To further demonstrate the potential of the BJ2A CVPs as a vaccine candidate, JEV-specific neutralizing antibodies were measured by PRNT, which provides a reasonable immunogenic correlation to protection (Chen et al., 2005). Neutralization efficacy was determined by PRNT 70 titer (serum dilution giving a 70% plaque reduction compared with plaque formation in virus-only controls). Seroconversion was defined as a fourfold or greater increase in PRNT 70 titer (WHO, 2005). Indeed, a fourfold increase of JEV-specific neutralizing antibody titers were detected in pooled sera from mice immunized with BJ2A CVPs (PRNT 70 = 1:160) than from those immunized with saline

(**Table 1**). The result suggested that the BJ2A CVPs could elicit effective immunity against JEV infections.

## DISCUSSION

### Circumventing the Epitope Size-Limitation Problems for Virus-Based Vector Systems by the Incorporation of FMDV 2A Peptide

The use of plants as safer and less expensive production systems for vaccine antigens has been actively investigated for more than 20 years (Rybicki, 2010), including several plant virus-based expression systems (for excellent reviews, see Lico et al., 2008; Rybicki, 2014; Chen, 2015; Streatfield et al., 2015; Shahid and Daniell, 2016). The CP genes of viruses are commonly exploited for the development of various strategies, since CP genes are usually expressed with high efficiency and provide natural scaffoldings for the target proteins to be displayed on the surface of CVPs (Lico et al., 2008). However, the production of antigens using plant viral vectors is hindered by several common limitations that stem from the interference of the normal biological functions of viral proteins by the fused peptides. These problems include: (1) the reliability of epitope presentation affected by the nature and sizes of foreign peptides (e.g., Bendahmane et al., 1999; Jiang et al., 2006; Uhde-Holzem et al., 2010; Zhang et al., 2010); (2) mutual restriction between encoding recombination virus RNA and the chimeric CP (e.g., Rao, 2006; Schneemann, 2006), and virus-host interactions (e.g., Porta et al., 2003; Ahlquist et al., 2005; Chen et al., 2007); (3) the stability of the foreign fragments over long-term successive passages (e.g., Porta and Lomonosoff, 1998; Porta et al., 2003; Lico et al., 2006); (4) reduced efficiency for virion assembly caused by special structural features of the chimeric CP (e.g., Canizares et al., 2005), and (5) the changes in virion morphology and stability due to cysteine residues in the foreign peptide (e.g., Li et al., 2007). Likewise, the construct pBJ, harboring direct fusion between JEV EDIII and BaMV CP, was not infectious, and the fusion protein was not detected in inoculated plants (**Figures 1C, 2**). In this study, we presented several lines of evidence that these obstacles were circumvented by the incorporation of FMDV 2A co-translational dissociation peptide in between JEV EDIII and BaMV CP. The resulting construct, pBJ2A, was infectious, and generated chimeric virus

progeny BJ2A which expressed two fusion proteins, EDIII-2A-CP and EDIII-2A, and one non-recombinant BaMV CP in plants (**Figures 1C**, left panel, **2**). The chimeric virus BJ2A could infect *N. benthamiana* systemically and produce JEV EDIII throughout whole plants (**Figure 1C**, right panel). The coding sequence of the foreign peptide EDIII-2A was stably maintained in the genome of the chimeric virus BJ2A after nine serial passages in *C. quinoa* leaves (**Figure 2**). The fusion protein EDIII-2A-CP and free-form BaMV CP subunits were able to assemble into filamentous BJ2A CVPs (**Figures 3A–C**). Although these JEV EDIII contain two cysteines, which potentially could cause changes in virion morphology and stability (Li et al., 2007), BJ2A CVPs exhibited the same particle morphology as that of the wild type BaMV's (**Figures 3A–C**). Furthermore, JEV EDIII antibody could specifically recognize BJ2A CVPs, indicating that the JEV EDIII peptide was properly presented on the surface of BJ2A CVPs (**Figures 3B,C**). Most importantly, BJ2A CVPs elicited immuno-responses in mice to generate neutralizing antibodies against the infection of JEV (**Figure 4** and **Table 1**).

Foot-and-mouth disease virus 2A peptide leads to partial dissociation of the fusion proteins with various efficiency for different fusion constructs (Donnelly et al., 2001). In this study, the incorporation of FMDV 2A peptide allowed production of enough free-form BaMV CP and the EDIII-2A-Cp fusion protein for both the assembly of stable CVPs and proper display of the epitope on the surface. In contrast, the CP produced by the construct pBJ is expected to be the EDIII-CP fusion form only, which might hinder the virion assembly process and result in the loss of infectivity of pBJ (as shown in **Figure 1**). The use of FMDV 2A peptide might have facilitated the virion assembly of the chimeric viruses *in planta* and likely contributed to the maintenance of the foreign coding sequences over long-term successive passages (**Figure 2**). Furthermore, the BaMV virion-based epitope-presentation system might provide an adjuvant-like function (Gerloni et al., 2000; Savard et al., 2011) and compensated for the partial incorporation of the EDIII-2A-CP in the CVPs.

The immunization assays in mice further confirmed that the EDIII peptide was presented in a biologically functional conformation on the surfaces of the CVPs, since the BJ2A CVPs elicited effective immuno-response against JEV infection in mice (**Figure 4** and **Table 1**). These results demonstrated the potential and applicability of the BaMV-based vector system in producing potent vaccine candidates in plants.

**TABLE 1 |** Plaque reduction neutralization titers of sera obtained from mice immunized with BJ2A chimeric virus particles.

| Immunogen | Plaque neutralization titers* |
|-----------|-------------------------------|
| Saline    | 1:40                          |
| BJ2A      | 1:160                         |
| rEDIII    | 1:320                         |

\*Represented as the serum dilution yielding 70% reduction in plaque number.

Pooled sera from six mice were used for the study. Sera from mice immunized with saline or rEDIII were used as negative and positive controls, respectively.

### Comparison with Other Plant-Based Vaccine Candidate Producing Systems against JEV or Related Viruses

As mentioned above, plants have been actively explored as effective vaccine candidate-producing systems in recent years. For JEV and related flaviviruses, transgenic and transient expression approaches have been documented (Martinez et al., 2012; Chen and Lai, 2013). JEV subunit vaccine candidate

produced in transgenic rice has been reported to elicit antigen-specific neutralizing antibodies in mice previously (Wang et al., 2009). However, the yields of JEV E protein expressed in the leaves of transgenic rice were relatively low, amounting to  $1.1\text{--}1.9 \mu\text{g mg}^{-1}$  (corresponding to 0.11–0.19% of TSP) (Wang et al., 2009). For transient expression approach, the EDIII of Dengue virus type 2 (D2EDIII), with only slight differences in structure from that of JEV EDIII (Chavez et al., 2010), was successfully produced in *N. benthamiana* using tobacco mosaic virus (TMV)-based duplicated-promoter strategy. The yield of D2EDIII protein accounted for 0.28% of TSP (Saejung et al., 2007). For another closely related virus, West Nile virus (WNV), Chen et al. (2011) developed a virus-like particle (VLP) vaccine by fusing the EDIII of WNV to the C-terminus of hepatitis B core antigen (HBcAg) and utilized a geminivirus-based vector to express the recombinant protein in *N. benthamiana* (Chen et al., 2011). The assembly of the VLP and the effectiveness in inducing strong B and T-cell responses were demonstrated. The yield of the WNV EDIII-HBcAg fusion protein was estimated to be  $\sim 0.35 \mu\text{g mg}^{-1}$  fresh leaf weight (FLW). By using the MagnICON vector system, the accumulation level was increased to  $>1 \mu\text{g mg}^{-1}$  FLW (Chen, 2015). In contrast, the BJ2A virus expressed JEV EDIII-2A-CP fusion proteins in the leaves at levels reaching  $8.9 \pm 4.3 \mu\text{g mg}^{-1}$  FLW. Therefore, the BaMV-based vector systems have enabled rapid expression of recombinant proteins at levels comparable to or higher than those produced with previous transgenic or other transient expression approaches in plants.

As for immunogenicity, the plant-made D2EDIII elicited only low level of anti-dengue virus antibodies, and no antibody induction was detected when mice were immunized without adjuvant (Saejung et al., 2007), possibly due to the small size of D2EDIII fragment expressed. In comparison, the BJ2A virus displayed the peptide of interest on the surface of assembled CVPs, enhancing immunogenicity (**Table 1**) by taking advantage of using BaMV CP as the dominant pathogen-derived antigens (Gerloni et al., 2000; Massa et al., 2008). VLPs and CVPs have been known to induce strong protective responses in the absence of adjuvants (Roldao et al., 2010). The repetitive display of the epitopes on the quasi-crystalline surface of CVPs may serve as the prime target for B-cell recognition and trigger strong B-cell responses (Fehr et al., 1998). It has been shown that the immunization by using JEV EDIII can elicit the generation of neutralizing antibodies to protect against JEV infection (Kaur et al., 2002). In this study, we have found that BJ2A CVPs could induce IgG-level immune responses in mice (**Figure 4A**). Moreover, the fluorescence staining results indicated that BJ2A CVPs successfully induced the anti-JEV virus antibody in mice (**Figure 4B**).

As for the preparation of immunogens, the D2EDIII proteins were purified by immobilized metal ion affinity chromatography (Saejung et al., 2007). In this study, the macromolecular nature of BJ2A CVPs allowed for the development of easy procedures for virion purification and the recovery of high doses of recombinant protein by simple centrifugation. Therefore, BaMV-based epitope presentation strategy provides an efficient

alternative for convenient, rapid, and low-cost expression of vaccine candidates.

## The Advantages of BaMV-Based Epitope Presentation System

Plants have been explored as bioreactors for the production of therapeutic proteins, and several plant-produced biopharmaceuticals have been through Phases II and III clinical trials in humans (Daniell et al., 2009; Rybicki, 2010, 2014; Thomas et al., 2011; Chen, 2015). It has also been shown that the plant-produced CVPs administered to animals intranasally, intraperitoneally or orally are able to induce strong neutralizing immune responses (Pogue et al., 2002; Rybicki, 2010). In addition, many achievements have been made using plant virus-based vector with FMDV 2A strategy for expressing foreign proteins as vaccines (e.g., Smolenska et al., 1998; O'Brien et al., 2000; Marconi et al., 2006; Zelada et al., 2006; Uhde-Holzem et al., 2010). In this study, we demonstrated that the BaMV-based vector system allowed expression of longer peptide, up to 111 amino acids, on CVPs than *Potato virus X*-based vector did (Marconi et al., 2006; Uhde-Holzem et al., 2010). The BaMV-based vector offered some advantages compared to other available systems. Firstly, BaMV has a narrow host range in nature, and therefore is ecologically safer for field use (Hsu and Lin, 2004), minimizing the concern for environmental contaminations. Secondly, by the incorporation of FMDV 2A peptide, BaMV-based epitope-presentation vector was stable over long-term successive passages, as opposed to the previously described systems (Porta and Lomonosoff, 1998; Porta et al., 2003; Lico et al., 2006). Thirdly, the plant, *C. quinoa*, used for the production of JEV subunit vaccine candidate is a widely cultivated crop (Bhargava et al., 2006), and poses minimal safety concern in animals. Furthermore, we have resolved the atomic model of the BaMV virion structure by using cryo-electron microscopy recently (DiMaio et al., 2015). This model provides the theoretical basis for the modeling of more candidate epitopes to be presented on BaMV-based vector system by using convenient *in silico* analyses.

## CONCLUSION

To our knowledge, this is the first report describing the production of a vaccine candidate of JEV EDIII using plant virus-based vector system. Our results also demonstrated the feasibility of using FMDV 2A peptide to circumvent some commonly encountered problems for plant virus-based epitope presentation systems. This strategy enabled the production of large quantity of both EDIII-2A-CP fusion protein and free CP in plant cells, allowing the self-assembly of stable CVPs using the two forms of CPs. As compared to the construct pBJ, which does not express non-recombinant BaMV CP, the incorporation of 2A peptide improved the infectivity of the chimeric virus BJ2A and might contribute to the enhanced stability over serial passages and the preservation of key structural features of the CVPs. The BaMV-based CVP vaccine successfully induced the generation of neutralizing antibodies

against JEV infection. Together, these results demonstrated that the BaMV-based CVP system may serve as an alternative for the production of effective and useful vaccine candidates against JEV infections.

## AUTHOR CONTRIBUTIONS

Designed the study: T-HC, C-CH, J-TL, N-SL, Y-LLi, and Y-HH. Analyzed the data: T-HC, J-TL, Y-WH, and Y-LLe. Wrote the manuscript: T-HC, C-CH, and Y-HH. Interpreted the data, and revised the manuscript: C-CH, Y-WH, N-SL, Y-LLi, and Y-HH. All authors read, edited and approved the final manuscript.

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**Conflict of Interest Statement:** 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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