



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 (CO₂) and bicarbonate (HCO₃⁻). The reaction of CO₂ + H₂O ↔ HCO₃⁻ + 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: α, β, γ, δ, and ε, 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 α, β, and γ classes, with the β 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 HCO₃⁻ for lipid biosynthesis (Hoang and Chapman, 2002) and the regulation of CO₂-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 OD₆₀₀ 0.8~1.0; the cells were collected by centrifugation at 5000 rpm, then suspended in the induction medium (10 mM MgCl₂, 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'-GTCCG 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 2.5×10^5 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 NH₄OAc, washed, dried, and dissolved in 30 µl de-ionized H₂O. 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 NH₄OAc and dissolved in 13 µl de-ionized H₂O.

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'-ACTGCCAATTGTCCCCTACA-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'-TGTGCTGAACGGGTATGAG-3') or BaMV+1766 (5'-CACATCCGGCACTTACCA-3') and BaMV-2002 (5'-ATGTATCACGGAAATAAGAGTT-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 MgCl₂, 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-ORF Fusion Protein

NbCA-ORF 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*HI and *Xho*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 $OD_{600} = 0.7 \sim 1.2$ (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_2PO_4 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 $-80^\circ C$ 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 $MgCl_2$, 120 mM KCl, 0.1% β -mercaptoethanol, 20% glycerol, 1 μ M 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 $500 \times g$ for 10 min to remove the cell debris. The pellet was resuspended in suspension buffer (50 mM Tris-HCl pH 8.2, 10 mM $MgCl_2$, 1 mM dithiothreitol, 1 μ M pepstatin A, 1 μ M leupeptin) after centrifugation at $30,000 \times g$ 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 μ M pepstatin A, 0.1 mM PMSF, 1 mM dithiothreitol) and centrifuged at $72,100 \times g$ 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 μ l of the replicase complex preparation (pH 8.0) was added to a total 50 μ l reaction containing 2 mM (A, C, and G) TP, 2 μ M UTP, 3 mM $MgCl_2$, 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 μ M [α - ^{32}P]UTP (3000 Ci mmol/l, Dupont-NEN) and 5.2 μ g 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 μ l 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 μ l of the replicase complex preparation was first treated with 10 units of micrococcal ribonuclease containing 2.5 mM Ca(OAc)₂ to remove endogenous RNA at 30°C for 30 min in a total volume of 11.5 μ l reaction. Then the reaction was terminated by adding 16 mM EGTA and set on ice for 1 ~ 2 min. An aliquot of 15 μ l mixture was subjected to a total 50 μ l 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).

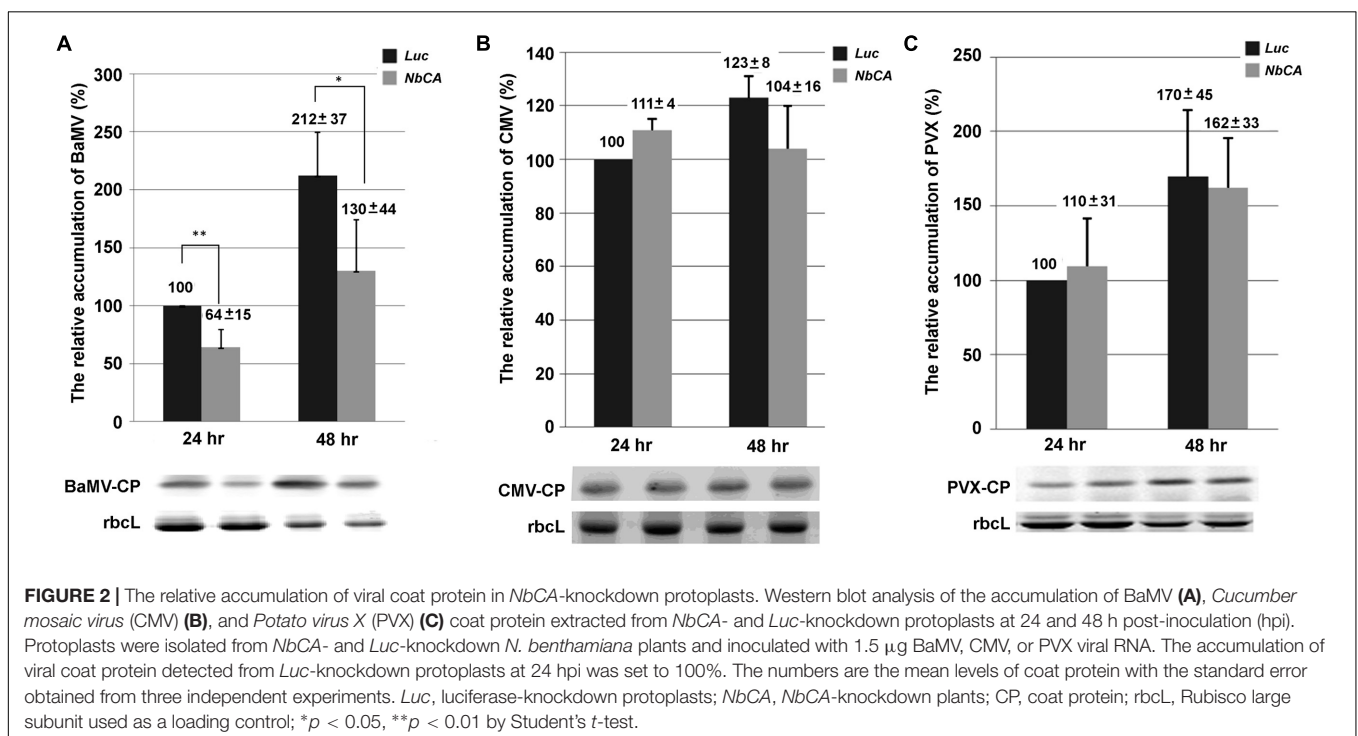
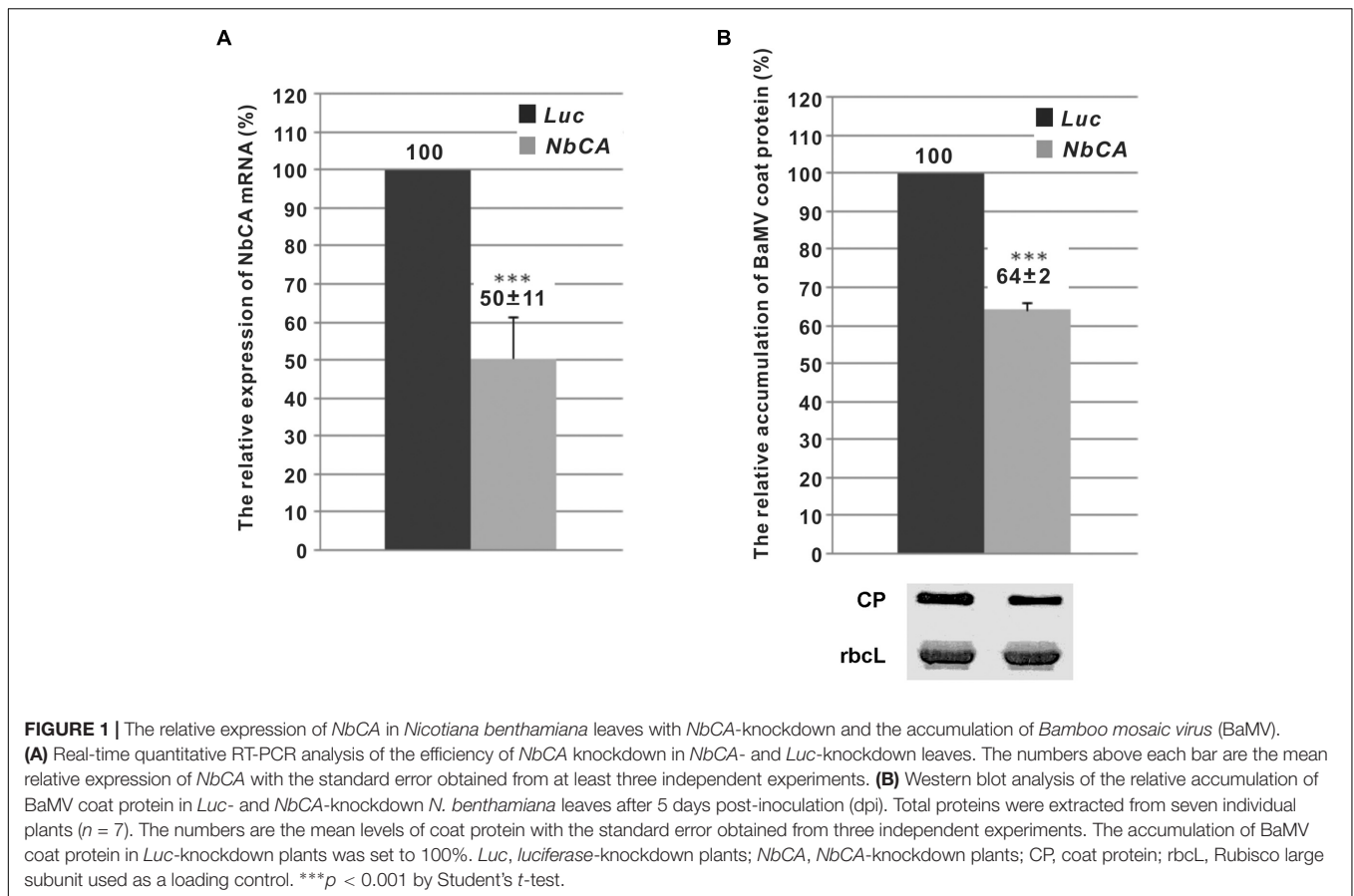
RNA Preparation

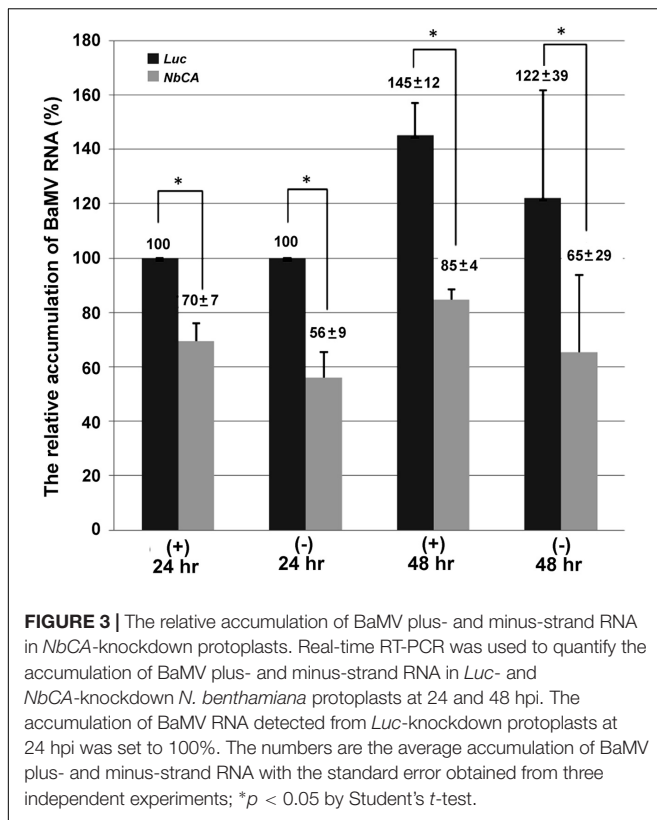
Ba-77 RNA (the 3'-end 77 nt of BaMV minus-strand RNA) and r138/40A RNA (the 3' UTR of BaMV RNA) was prepared in an *in vitro* transcription with plasmids constructed previously (Cheng et al., 2001). The reaction was carried out in 100 μ l containing 10 μ g linearized plasmid (*Eco*RI and *Bam*HI for Ba-77 and r138/40A, respectively, in pUC18), 40 mM Tris-HCl pH 8.0, 2 mM spermidine, 8 mM $MgCl_2$, 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 $-80^\circ C$.

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.





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; Bombarely 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

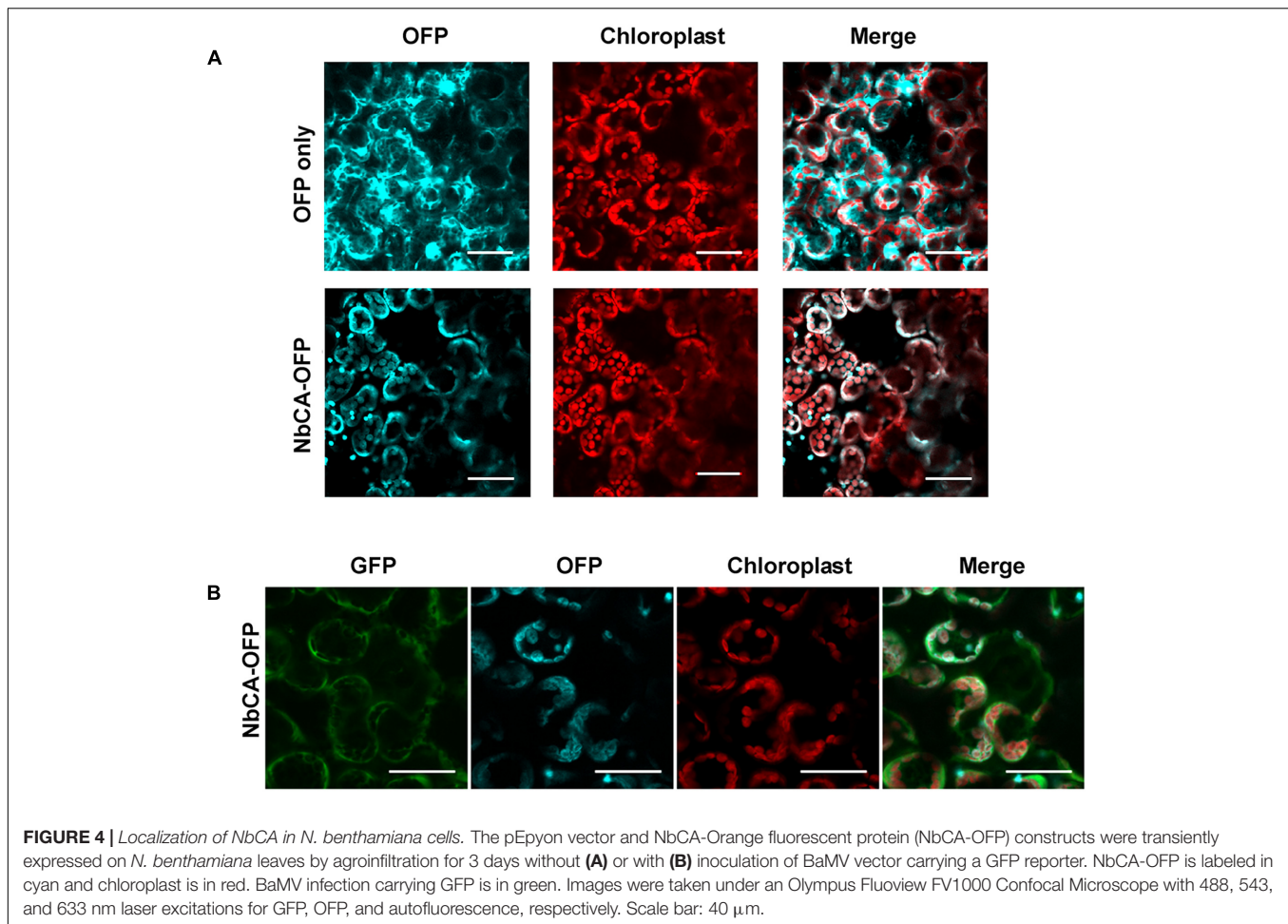


FIGURE 4 | Localization of NbCA in *N. benthamiana* cells. The pEpyon vector and NbCA-Orange fluorescent protein (NbCA-OFP) constructs were transiently expressed on *N. benthamiana* leaves by agroinfiltration for 3 days without (A) or with (B) inoculation of BaMV vector carrying a GFP reporter. NbCA-OFP 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 μ 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 templates 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 CO₂ into bicarbonate for carbon fixation

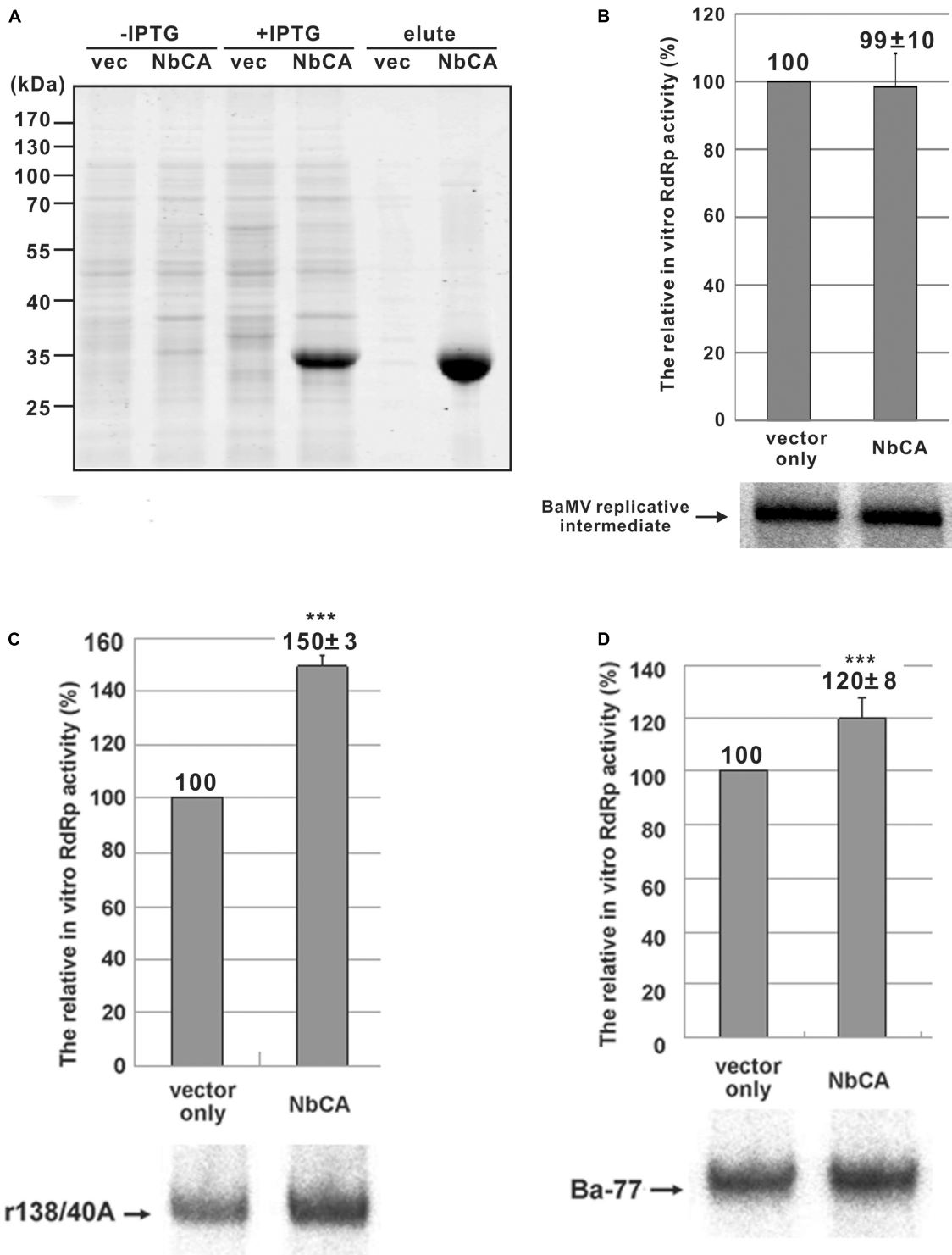
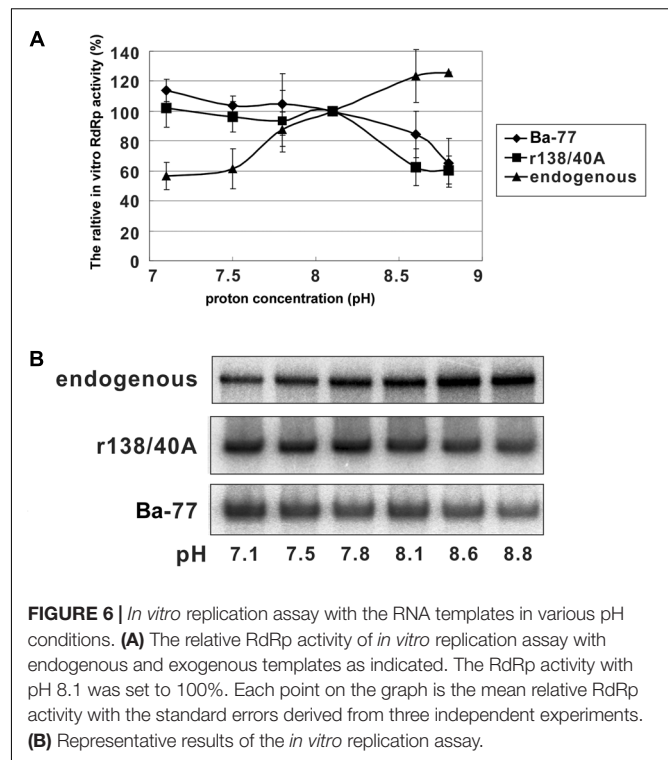


FIGURE 5 | The expression of NbCA in *Escherichia coli* and *in vitro* replication assays with endogenous and exogenous templates. **(A)** Total proteins were extracted from *E. coli* that expressed vector only (vec) or NbCA with or without the induction of IPTG as indicated, separated on a 12% polyacrylamide gel/SDS, and stained with Coomassie blue. The eluents 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, β -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 CO₂ 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 Δ *NCE103*, the *Saccharomyces cerevisiae* CA-like gene deletion strain sensitive to an oxidized environment such as in the presence of H₂O₂; 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 H₂O₂-degrading activity (Chen et al., 1993a,b; Conrath et al., 1995). Accordingly, SABP3/*NtCA* or *NbCA* may also have antioxidant ability to degrade H₂O₂ 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 potyvirus, 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



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⁺ oxidoreductase (FNR). FNR transfers electrons from the reduced form of ferredoxin (Fd) to NADP⁺ 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.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2017.02046/full#supplementary-material>

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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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