An E3 ubiquitin ligase from Nicotiana benthamiana targets the replicase of Bamboo mosaic virus and restricts its replication

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SUMMARY

One up-regulated host gene identified previously was found involved in the infection process of Bamboo mosaic virus (BaMV), a single-stranded positive-sense RNA virus. The full length cDNA of this gene was cloned by 5’ and 3’-rapid amplification of cDNA ends and found to encode a polypeptide containing a conserved really interesting new gene (RING) domain and a transmembrane domain. The gene might function as an ubiquitin E3 ligase. We designated this protein in Nicotiana benthamiana as ubiquitin E3 ligase containing RING domain 1 (NbUbE3R1). Further characterization by using Tobacco rattle virus-based virus-induced gene silencing (loss-of-function) revealed that increased BaMV accumulation was in both knockdown plants and protoplasts. The gene might have a defensive role in the replication step of BaMV infection. To further inspect the functional role of NbUbE3R1 in BaMV accumulation, NbUbE3R1 was expressed in N. benthamiana plants. The wild-type NbUbE3R1-orange fluorescent protein (NbUbE3R1-OFP), NbUbE3R1/ΔTM-OFP (removal of the transmembrane domain) and NbUbE3R1/mRING-OFP (mutation at the RING domain, the E2 interaction site) were transiently expressed in plants. NbUbE3R1 and its derivatives all functioned in restricting the accumulation of BaMV. The common feature of these constructs was the intact substrate-interacting domain. Yeast two-hybrid and co-immunoprecipitation experiments used to determine the possible viral-encoded substrate of NbUbE3R1 revealed the replicase of BaMV as the possible substrate. In conclusion, we identified an up-regulated gene, NbUbE3R1 that plays a role in BaMV replication.

Keywords: Bamboo mosaic virus, E3 ubiquitin ligase, Nicotiana benthamiana, protein-protein interaction, RNA-dependent RNA polymerase, viral RNA replication, yeast two-hybrid.

INTRODUCTION

Plants have evolved various strategies to face rapid environmental changes and pathogen invasions (Boller and Felix, 2009; Cohn et al., 2001). Recognizing and confronting these pathogens are involved in regulating the massive gene expression involved in plant–pathogen interactions (Hou et al., 2009). Ubiquitin-mediated protein degradation is widely used amongst eukaryotes. Many ubiquitin-related genes in Arabidopsis thaliana have been characterized. Biochemical and proteomic studies revealed that the ubiquitin proteasome system (UPS) plays central roles in many processes of plants (Alcaide-Loridan and Jupin, 2012; Bachmair et al., 2001; Vierstra, 2009). Plants exploit the UPS as a major regulatory process in hormone signalling, regulation of chromatin structure and transcription, tailoring morphogenesis, response to environmental challenges, and self-recognition and attacking pathogens (Vierstra, 2009).

In the UPS process, ubiquitin is first activated by the ATP-dependent ubiquitin-activating enzyme (E1). The activated ubiquitin is then transthiolated to ubiquitin-conjugation enzyme (E2). Ubiquitin E3 ligase (E3), the key component for UPS targeting specificity, interacts with E2-ubiquitin and the target protein to facilitate the transfer of ubiquitin and thus generate the specificity of ubiquitin modification (Metzger et al., 2014; Yuan et al., 2013).

On the basis of their composition and the activation mechanisms, four main types of E3 ligases are known in plants: (i) homologous to the E6AP carboxyl terminus (HECT), (ii) really interesting new gene (RING), (iii) U-box, and (iv) cullin (a molecular scaffold protein)-RING ligases (CRLs) (Vierstra, 2009). The HECT type of E3 ligase is activated by transthiolation with ubiquitin, which is then conjugated to the substrate. RING or U-box E3 ligase mediates the transfer of ubiquitin from E2 directly to the substrate. The RING domain of E3 chelates two Zn^{2+} ions in a cross-brace structure with octet Cys and His residues used as a platform for E2 binding. RINGs are composed of one or two His residues, C3H2C3 (RING-H2) or C3HC4 (RING-HC), and other variations (Metzger et al., 2014). The U-box exploits the electrostatic interaction to stabilize
the ubi-E2 binding pocket (Vierstra, 2009). The CRLs consist of a cullin and a RING-containing domain, RING-BOX 1, that could bind the ubi-E2 and various adaptors and recognize target proteins (Hua and Vierstra, 2011). The target proteins have different fates depending on the type and extent of the ubiquitination.

Unusual expression levels of ubiquitin and E1 and/or E2 enzyme in the UPS pathway were demonstrated to have a comprehensive effect on cell reprogramming in the plant defence system. The E3 ligases involved in plant–pathogen and gene–gene interactions result in induction of disease resistance (Delauré et al., 2008; Zeng et al., 2006). Furthermore, the UPS was shown to have a resistance role in viral invasion (Takizawa et al., 2005) or to be involved in specific gene-mediated resistance responses (Dielen et al., 2011). For example, interfering in the ubiquitin-conjugation pathway could induce different plant responses to infection with Tobacco mosaic virus, which supports that the UPS participates in the virus–host interaction (Alcaide-Loridan and Jupin, 2012; Becker et al., 1993). The movement proteins (MPS) of Tobamovirus, which facilitate viral cell-to-cell transport, were found to be polyubiquitinated and degraded by 26S proteasome (Reichel and Beachy, 2000). The 66 kDa RNA-dependent RNA polymerase (RdRp) of Turnip yellow mosaic virus (TYMV) was targeted by the UPS in infected plant cells and down-regulated viral replication (Camborde et al., 2010). These findings reveal the link between the plant UPS and viral pathogens; however, the detailed mechanism is still unknown (Alcaide-Loridan and Jupin, 2012).

Bamboo mosaic virus (BaMV) infects more than 90% of bamboo plants. The virus induces yellow mosaic patterns on leaves and brown streaks on shoots and young culms, which causes a strong taste of shoots and results in decreased quality and output of bamboo. BaMV is believed to be the largest obstacle in the production of bamboo in Taiwan (Hsu et al., 2000). BaMV, a flexuous-rod virus composed of coat protein (CP) and genomic RNA, belongs to the Potexvirus genus of the Flexiviridae family (Lin et al., 1977). The genome of BaMV is 6.4 kb long and has a 5′ m7GpppG structure and a 3′ poly (A) tail containing five conserved open reading frames (ORFs) (Lin et al., 1994). ORF1 encodes a 155 kDa polypeptide containing three functional domains: the capping enzyme domain, with GTP methyltransferase and 5′-adenosylmethionine (AdoMet)-dependent guanylyltransferase enzyme activities (Huang et al., 2004; Li et al., 2001a, b); the helicase-like domain, with nucleoside triphosphatase and RNA 5′-triphosphatase activities (Li et al., 2001b); and a RdRp domain that recognizes the 3′-untranslated region of the BaMV genome and is involved in viral RNA replication (Huang et al., 2001; Li et al., 1998). ORFs 2-4 encode three overlapping MPS (termed triple gene block, TGB) for viral cell-to-cell movement (Chen et al., 2012). ORF5 encodes a 25 kDa viral capsid protein required for viral RNA encapsidation (Chen et al., 2010, 2012). Two major subgenomic RNAs of approximately 1 kb and 2 kb were found in infected cells (Tsai et al., 1999).

The defence mechanism in Nicotiana benthamiana was suggested to be compromised (Christie and Crawford, 1978) and susceptible to numerous viral pathogens (Bombarely et al., 2012). Therefore, N. benthamiana plants have been exploited to mature the virus-induced gene silencing (VIGS) technology and explore RNA interference mechanisms (Angell and Baulcombe, 1997; Baulcombe, 1999, 2004; Kumagai et al., 1995). N. benthamiana is widely used as a model plant for research on plant–microbe interactions (Goodin et al., 2008). Previously, our laboratory used the cDNA-amplified fragment length polymorphism ( AFLP) technique to screen and identify BaMV infection-associated genes in N. benthamiana (Cheng et al., 2010). VIGS was then used to knockdown gene expression in N. benthamiana for further investigation. Amongst gene fragments up-regulated in N. benthamiana, a serine/threonine kinase-like (NbSTKL) of N. benthamiana was shown to facilitate cell-to-cell movement (Cheng et al., 2013). A new tau group of the GTS gene, NbGSTU4, was demonstrated to bind the 3′ UTR of BaMV RNA and enhance viral RNA replication in vitro (Chen et al., 2013). Amongst other up-regulated gene fragments, ACGT2-1 was further studied in this work.

The aim of this study was to uncover the role of ACGT2-1 in the infection cycle of BaMV. We isolated the full length ACGT2-1 cDNA clone, which encodes a RING-containing protein of N. benthamiana and may play a key role in the ubiquitination pathway.

RESULTS

The gene containing ACGT2-1 is a putative C3H2C3-type zinc finger ubiquitin E3 ligase

The expression profile of the up-regulated gene containing the ACGT2-1 cDNA fragment identified by cDNA-AFLP (Cheng et al., 2010) was further confirmed by Reverse Transcription-Polymerase Chain Reaction (RT-PCR). The expression of the gene containing ACGT2-1 was up-regulated at 5 dpi to 7 dpi after BaMV inoculation (Fig. S1).

To identify the gene containing ACGT2-1 cDNA fragment, 3′ and 5′ rapid amplification of cDNA ends (RACE) was performed. The full length cDNA obtained from RACE was about 1.7 kb. The coding region was 1218 bp and encoded a 406-amino acid polypeptide (Fig. S2). The sequence of the in silico translated polypeptide was predicted to contain a transmembrane domain, a RING zinc finger domain and a C-terminal coiled-coil region by domain architecture analysis (Fig. S2). The results derived from the sequence comparison by using the available databases indicated that the RING finger domain is highly conserved.
commonly found in E3 ubiquitin ligases, and plays a key role in the ubiquitination pathway (Guzman, 2012). Therefore, the up-regulated gene containing the ACGT2-1 fragment could be a putative C3H2C3-type zinc finger E3 ubiquitin ligase. We then designated this polypeptide ubiquitin E3 RING type ligase 1 of N. benthamiana, NbUbE3R1.

The up-regulated gene NbUbE3R1 is involved in BaMV infection

To investigate whether NbUbE3R1 is involved in the BaMV infection cycle, we used Tobacco rattle virus (TRV)-based VIGS to knockdown its expression in N. benthamiana plants. To rule out the possibility of an off-target by using the ACGT2-1 fragment to knockdown the gene expression, the sequence of ACGT2-1 was analysed in the N. benthamiana sol genome sequence database (Fernandez-Pozo et al., 2015). We did not find any sequence matching that of ACGT2-1 with consecutive residues longer than nt 20 besides the target gene, NbUbE3R1. Therefore, using the sequence of ACGT2-1 to knockdown the expression of NbUbE3R1 in N. benthamiana could be specific.

At approximately 10 days to 14 days post-agroinfiltration, the morphology of NbUbE3R1-knockdown plants showed no obvious difference from Luciferase-knockdown plants (Fig. S3). The knockdown efficiency measured by real-time RT-PCR was reduced 27% in NbUbE3R1-knockdown plants as compared with control plants (Fig. 1A). The accumulation of BaMV CP in NbUbE3R1-knockdown plants quantified at 5 dpi was significantly increased 1.34-fold that of control plants (Fig. 1B). Therefore, the target gene was involved in the infection cycle of BaMV and possibly plays a defence role against BaMV infection.

NbUbE3R1 is involved in BaMV RNA replication

To inspect how NbUbE3R1 affects the accumulation of BaMV in N. benthamiana, protoplasts derived from the NbUbE3R1-knockdown plants were isolated and inoculated with viral RNA. The accumulation of BaMV CP was increased to 291% of that of control protoplasts at 24 h post-inoculation (Fig. 1C). The accumulation of viral RNA in NbUbE3R1-knockdown protoplasts was significantly increased 2.10- and 2.35-fold for the plus- and minus-strand RNA, respectively, that of control protoplasts (Fig. 1D and E). These results suggest that the ACGT2-1-containing gene NbUbE3R1 might be involved in viral RNA synthesis.

Subcellular localization of NbUbE3R1

Because NbUbE3R1 contains a transmembrane domain, it is likely a membrane-associated protein. Therefore, we further analysed NbUbE3R1 in the plant membrane protein database Aramemnon to predict its possible role and found that NbUbE3R1 could be involved in the secretory pathway. To experimentally determine the subcellular location of NbUbE3R1, orange fluorescent protein (OFP)-tagged NbUbE3R1 or OFP alone was transiently expressed in N. benthamiana leaf and protoplasts. On confocal microscopy, wild-type NbUbE3R1-OFP clustered to form speckled spots in protoplasts (Fig. 2), whereas localization of the mutant with the N-terminus transmembrane truncation, NbUbE3R1/∆TM-OFP, was similar to that with OFP alone.

NbUbE3R1 negatively regulates BaMV infection

The results from NbUbE3R1-knockdown experiments suggested that NbUbE3R1 could have a defensive role against BaMV. To verify this hypothesis, NbUbE3R1-OFP and its derivatives were expressed in N. benthamiana leaves and BaMV was inoculated in NbUbE3R1-expressed leaves. The accumulation of BaMV of control plants expressing OFP only (Fig. 3). To determine whether the membrane association of NbUbE3R1 is critical for its defensive role, we expressed NbUbE3R1/∆TM-OFP in leaves. The accumulation of BaMV CP was significantly reduced to 31% of that of the control. Therefore, NbUbE3R1 without the transmembrane domain could still have a significant role against BaMV. However, the wild-type NbUbE3R1 containing the transmembrane domain was more efficient in targeting BaMV.

Because NbUbE3R1 is predicted to be an E3 ubiquitin ligase, the RING domain may contain an E2 binding site required for activity to target the substrate for degradation. To validate this hypothesis, a mutant with five-point mutations (S166A, V167A, W196A, P204A and L205A) in the RING finger domain of NbUbE3R1 was constructed (Fig. S2) and designated NbUbE3R1/mRING. These residues in the RING domain of E3s were reported to be potential E2-contact points (Deshaies and Joazeiro, 2009). NbUbE3R1/mRING carrying these mutations is expected to be defective in RING–E2 interaction to a certain extent, so the association of NbUbE3R1 catalytic activity and BaMV replication can be revealed. The accumulation of BaMV in NbUbE3R1/mRING-OFP-expressed leaves was approximately 35% of that of the control (Fig. 3). Therefore, NbUbE3R1 restricting BaMV replication does not need to have the full function of E3 ligase. Similarly, the reduced BaMV replication of NbUbE3R1/mRING–OFP was significantly less than that of the wild type (Fig. 3). These results suggested that the catalytic activity of NbUbE3R1 does play a significant role in reducing the accumulation of BaMV. Thus, the transmembrane and RING finger domains of NbUbE3R1 could also play in part in negative regulation of BaMV infection.

NbUbE3R1 protein is unstable in vivo

Transiently expressed NbUbE3R1-OFP and NbUbE3R1/mRING-OFP were barely detected by Western blot analysis (Fig. 4) perhaps because of the instability of NbUbE3R1, which might
undergo auto-ubiquitination or be targeted by an endogenous degradation pathway (de Bie and Ciechanover, 2011). To clarify whether the low expression of NbUbE3R1 was due to the instability from the auto-ubiquitination of NbUbE3R1, plants were treated with the 26S proteasome inhibitor MG132 after transient expression of NbUbE3R1 and its derivatives. The expression of NbUbE3R1 was elevated by MG132 treatment (Fig. 4). Also, the expression of NbUbE3R1/mRING was higher than that of NbUbE3R1 perhaps because NbUbE3R1/mRING lost its ubiquitination activity and could not undergo self-regulated auto-ubiquitination within the plant cell.

Fig. 1  Relative expression and accumulation of NbUbE3R1 and BaMV in NbUbE3R1-knockdown Nicotiana benthamiana. (A) Relative expression of NbUbE3R1 in plants determined by real-time Reverse Transcript-Polymerase Chain Reaction (RT-PCR). The expression of actin was used for normalization. (B) The relative accumulation of BaMV coat protein (CP) in plants harvested at 5 days post-inoculation (dpi) determined by Western blot analysis. The expression of RuBisCo large subunit (rbcL) was the loading control. Protoplasts were isolated from Luciferase- or NbUbE3R1-knockdown plants and inoculated with BaMV viral RNA. The accumulation of BaMV CP (C) and viral RNA, plus-strand RNA (D) and minus-strand RNA (E), in plants harvested at 24 h post-inoculation quantified by Western and Northern blot analyses. The expression of rbcL and the ribosome RNA (rRNA) was the loading control in Western and Northern blot analyses, respectively. The expression in Luciferase-knockdown plants was set to 100%. The number above the statistical bar or above the blots is the mean ± standard error (SE) of three independent experiments. *P < 0.05, **P < 0.01 and ***P < 0.001 by Student’s t-test.

NbUbE3R1 interacts with BaMV replicase

The results from the transient expression experiments (Fig. 3) indicated that NbUbE3R1/mRING without the E3 ligase activity could still suppress BaMV replication, perhaps because the NbUbE3R1/mRING could still interact with the substrate (the BaMV replication-related protein). To elucidate the possible substrate of NbUbE3R1, we used a yeast two-hybrid assay to test whether NbUbE3R1 could interact with viral replicase. Each domain of the BaMV-encoded replicase was cloned into the pLEXA as a bait and designated pLEXA/Capping, -/Helicase, and -/RdRp.
The coding sequence of NbUbE3R1/ΔTM (a soluble form and with suppression activity) was cloned into pYESTrp2 as a prey and then transformed into yeast containing each domain of the viral replicase. Double transformants (yeast with both pLEX and pYES) were subjected to strong selective conditions. NbUbE3R1/ΔTM could interact with the RdRp domain in the yeast cells (Fig. 5).

Co-immunoprecipitation was used to validate the results of the yeast two-hybrid experiments. Agroinfiltrated N. benthamiana leaves were used to examine the interaction between NbUbE3R1 and BaMV replicase in planta. NbUbE3R1/ΔTM-OFP or OFP (a negative control) was co-infiltrated with BaMV/Rep-HA, an infectious cDNA clone with the replicase tagged with HA at its C-terminus. Total proteins were immunoprecipitated with anti-OFP magnetic beads. The replicase of BaMV could co-precipitate with NbUbE3R1/ΔTM (Fig. 6).

**DISCUSSION**

The study of differentially expressed genes after virus infection is a strategy for identifying host factors involved in virus infection...
cycles. We used a cDNA-AFLP technique to identify a number of these genes (Cheng et al., 2010) and revealed their involvement in BaMV infection (Huang et al., 2017a). One of the host factors in this study was demonstrated to play a defensive role against BaMV replication. Sequence analysis indicated that this gene contains a C3H2C3-type RING finger domain and is possibly an E3 ubiquitin ligase. The structure of the RING-H2 domain in NbUbE3R1 is similar to that of Arabidopsis Tóxicos en Levadura 54 (ATL54), which was demonstrated to harbour in vitro ubiquitin ligase activity (Noda et al., 2013). Therefore, we proposed that NbUbE3R1 could mediate a degradation or modification of BaMV replication-related protein.

Bioinformatics analysis indicated that NbUbE3R1 could be in the secretory pathway. We used OEP-tagged NbUbE3R1 to inspect the subcellular localization. However, the expression of this protein was so low and barely detected on Western blot analysis perhaps because NbUbE3R1 was unstable and might undergo auto-ubiquitination and self-destruction (Lin et al., 2008). We used MG132, a proteasome inhibitor, to treat leaves before infiltration, but we still could not obtain strong fluorescent signals. The expression experiments indicated that NbUbE3R1 associated with membrane or not could target BaMV and reduce its accumulation. However, NbUbE3R1 targeting to the membrane could be critical and more efficient against BaMV infection. Mutant NbUbE3R1/ΔTM-OFP with failure to localize at the membrane was less efficient in reducing the accumulation of BaMV.

The results of yeast two-hybrid and co-immunoprecipitation assays suggested that the RdRp domain of BaMV replicase could be one of the possible substrates of NbUbE3R1. We are missing the link of how and where NbUbE3R1 targets BaMV replicase. The availability of BaMV replicase for targeting could be in chloroplasts, where viral RNA replication occurs (Cheng et al.,...
Fig. 5 Interaction of NbUbE3R1/ΔTM with BaMV replicase in yeast cells. Yeast strain L40 co-transformed with the indicated plasmids was subjected to ten-fold serial dilution and incubated with minimal medium lacking tryptophan and histidine supplemented with 3-AT (5 mM) to identify protein interactions. Yeast containing pYES-Hsp90 and pLEX-RdRp was a positive control; yeast containing the vector pHybLex/Zeo and pYESTrp2 was a negative control.
in the cytoplasm, where replicase is translated or in the membrane-associated movement complex. Although NbUbE3R1 containing the transmembrane domain and the membrane-associated character was most efficient in reducing BaMV replication, a small portion of NbUbE3R1 transported to the chloroplasts could not be excluded.

The E3 ubiquitin ligases of the UPS were found involved in disease resistance as regulators of the plant defence responses (Azevedo et al., 2002; Berrocal-Lobo et al., 2010; Verchot, 2014). The replication enzyme p92 of Tomato bushy stunt virus (TBSV) was found regulated by the UPS (Barajas et al., 2009; Li et al., 2008). The ubiquitination of TBSV p33 could interrupt the interaction with the host protein Vps23p endosomal complexes required for transport (ESCRT), with failure in viral RNA replication (Barajas and Nagy, 2010). Also, the 66 kDa RdRp of TYMV was targeted by the UPS in infected plant cells and down-regulated viral replication (Camborde et al., 2010).

The UPS could also affect viral cell-to-cell movement. Increasing evidence has shown that viral MPs are regulated by proteasome turnover. The 69 kDa MP of TYMV could be polyubiquitinated and degraded by proteasome (Drugeon and Jupin, 2002; Verchot, 2014). The 17 kDa MP of polerovirus, TGB3 of PVX, and the 30 kDa MP and CP of Tobacco mosaic virus were regulated by the UPS degradation pathway (Jockusch and Wiegard, 2003; Ju et al., 2008; Mas and Beachy, 1999; Pazhouhandeh et al., 2006; Verchot, 2014). The CP of HIV type 1 was targeted by TRIM5 (the tripartite motif protein), which exhibits E3 ubiquitin ligase activity, and was removed by proteasome-dependent degradation (Li et al., 2013). This type of protein turnover may represent an antiviral defence mechanism (Hwang et al., 2011). These studies suggest that the UPS may target viral protein to interfere in viral invasion. In this study, we have identified an E3 ubiquitin ligase in N. benthamiana, which has not been reported before and could target the replicase of BaMV, which resulted in down-regulating BaMV replication.

**EXPERIMENTAL PROCEDURES**

**Plants and growth conditions**

*N. benthamiana* plants were grown in a growth room with 16-h day length at 28 °C.

**mRNA purification and quantification**

Total RNA was extracted from mock- or 500 ng BaMV virion-inoculated *N. benthamiana* plants by using Tripure isolation reagent (Roche, Germany) according to the manufacturer’s instructions. RNA of approximately 75 μg was incubated at 65 °C for 2 min and mixed with Dynabeads oligo(dT)25 for 5 min. The reaction was placed in the Dynabeads Magnetic Particle Concentrator (MPC) for 30 sec, and the supernatant was removed. The beads with poly(A)+ RNA were washed twice with a washing buffer (10 mM Tris-HCl, pH 7.5, 1 M LiCl and 2 mM EDTA), incubated with 10 μl ddH2O at 80 °C for 2 min, and placed in the MPC. The poly(A)-tailed mRNAs in water were transferred to a new tube.

Approximately 1 μg mRNA was mixed with 5 μM Oligo(dT)20 primer, incubated at 70 °C for 5 min and kept on ice. The reaction contained the pre-incubated mRNA, 3 mM MgCl2, 0.5 mM dNTP, 6 Us RNaseOUT (RNase inhibitor, Invitrogen, Carlsbad, CA, USA), and 5 Us reverse transcriptase (Promega, Madison, WI, USA).

To confirm the expression profile of ACGT2-1 after BaMV infection, primer sets GT2-1/F1 (5′ TAAGTGTAA-ACATGATGTTCCAAAT3′) and GT2-1/R1 (5′ CGAACCTATAACCTCCTGAT3′) and actin F (5′ GTGGTTTCATGAATGCCAGCA3′) and actin R (5′ GATGAAGATACTCACAGAAAGA3′) were used in PCR to detect the expression of NbUbE3R1 and β-Actin, respectively.

The expression of *NbUbE3R1* was analysed by quantitative RT-PCR (qRT-PCR) with the KAPA SYBR FAST qPCR Kit Master Mix (KapaBiosystems, Boston, MA, USA). The expression of actin was used for normalization. The reactions were set at thermal cycling conditions with 95 °C for 3 min, and 40 cycles at 95 °C for 3 sec followed by 60 °C for 20 sec. The specific primers for qRT-PCR were GT2-1/F1 and GT2-1/R4 (5′ GTTCTTGGAGAATAAAGTGCAC′).
Constructs

The cDNA fragment (ACGT2-1) (Cheng et al., 2010) was released from pGEM-T Easy vector (Promega, Madison, WI, USA) by digestion with EcoRI and cloned into the TRV2 vector for knockdown experiments. 3′ rapid amplification of cDNA ends (RACE) involved reverse transcription of mRNAs isolated from healthy N. benthamiana plants by using the primer 3′ RACE/RT (5′CAAACTGAGGCCGGATCCCTATATNN3′). Amplification involved two PCR reactions with one forward primer GT2-1/F1 and two reverse primers: 3′RACE/R1 (5′CAAACGTGACGGGGATCTAGATAG3′) and 3′RACE/R2 (5′GAGCCGGATCCCTATATGCGTGGGTTTG3′) were used in PCR. The final products were gel-purified and diluted 10-fold (5′). For 5′ RACE, the first-strand cDNAs were synthesized by using the SMARTer RACE cDNA Amplification Kit (Clontech Laboratories, Mountain View, CA, USA) according to the manufacturer’s protocol. Primers 5′RACE/long (5′CTAATAGACTCTACTATGG3′) and 5′RACE/R1 (5′CAGCCGATGACTAAGCTCCCGCTATAGCGTGGC3′) were used in PCR. The products were then diluted 10-fold and used as the template. The primer set 5′RACE/short (5′CTAATAGACTCTACTATGG3′) and 5′RACE/R2 (5′GGGCCAGATCACAAGGGTTTATTGTACG3′) was used in the second round of PCR. The final products were gel-purified and cloned into the pGEM-T Easy vector (Promega).

The ORF of NbUbE3R1 was PCR-amplified with the primer set GT2-1/F5 (5′GGTGCTAAGTGCCATTCTTATAG3′) (XbaI site underlined) and GT2-1/R5 (5′GGTACCATGGTTGATCATCCCAATTTGGTATATACGTACG3′) (KpnI site underlined). The amplified DNA fragment was cloned into the pEpyon/mOrange2 vector with XbaI and KpnI sites after sequence verification. The ORF of NbUbE3R1 was PCR-amplified with the primer set GT2-1/F5 (5′GGTGCTAAGTGCCATTCTTATAG3′) (XbaI site underlined) and GT2-1/R5 (5′GGTACCATGGTTGATCATCCCAATTTGGTATATACGTACG3′) (KpnI site underlined). The amplified DNA fragment was cloned into the pEpyon/mOrange2 vector with XbaI and KpnI sites after sequence verification.

The mutant NbUbE3R1/mRING-OPF was created by three cloning steps. First, the ORF of NbUbE3R1 was amplified with two sets of primer pairs, ME3F1 (5′GGCGTAAGATCACACAGAATTGGCGACGCPTGCGCCTCC3′) and ME3R1 (5′GGTGCGATGATATTATTGGCAGCGTGAATACTCC3′) was cloned into the pEpyon/mOrange2 vector. The mutant construct with removal of the transmembrane domain NbUbE3R1/ΔTM-OPF was created by PCR with the primer set GT2-1/F4 (5′GGTCTAGAATTTGGTATAGCCCAATTTTG3′) (XbaI site underlined) and GT2-1/R5. The amplified fragment was cloned into the pGEM-T Easy vector and transferred to the pEpyon/mOrange2 vector with XbaI and KpnI sites after sequence verification.

The mutant NbUbE3R1/mRING-OPF was created by three cloning steps. First, the ORF of NbUbE3R1 was amplified with two sets of primer pairs, ME3F1 (5′GGCGTAAGATCACACAGAATTGGCGACGCPTGCGCCTCC3′) and ME3R1 (5′GGTGCGATGATATTATTGGCAGCGTGAATACTCC3′) was cloned into the pEpyon/mOrange2 vector. The mutant construct with removal of the transmembrane domain NbUbE3R1/ΔTM-OPF was created by PCR with the primer set GT2-1/F4 (5′GGTCTAGAATTTGGTATAGCCCAATTTTG3′) (XbaI site underlined) and GT2-1/R5. The amplified fragment was cloned into the pGEM-T Easy vector and transferred to the pEpyon/mOrange2 vector with XbaI and KpnI sites after sequence verification.

The silenced leaves at 14 days post-agroinfiltration collected for analysis of specific gene knockdown were sliced into thin strips.
Leaves were digested with enzyme solution (0.1% bovine serum albumin, 0.6 mg/mL pectinase and 12 mg/mL cellulose in 0.55 M mannitol-MES pH 5.7) in the dark at 25 °C for 10 h–12 h. The extracted protoplasts were filtered by using a Miracloth and separated by centrifugation at 300 rpm for 7 min (KUBOTA KS-5000). Healthy protoplasts were further isolated as described (Chen et al., 2018). Approximately 2.5 × 10^5 protoplasts were inoculated with 1 μg BaMV viral RNA in 20% polyethyleneglycol (PEG) 6000. Inoculated protoplasts were resuspended in culture medium and incubated at 25 °C for 24 h under constant light. Total protein and RNA were extracted according to previously described protocols.

**Northern blot assay**

Approximately 1 μg RNA was mixed with 10 mM phosphate buffer, pH 7.0, 50% DMSO, 1 M glyoxal in a final 12 μl-reaction and incubated at 50 °C for 1 h. After denaturation, RNA was size-fractionated in an 1% agarose gel and blotted onto a nylon membrane (Hybond-N+, GE Healthcare, UK) by using 0.2 M NaOH transferring solution for 40 min. RNA was cross-linked to the membrane by UV exposure (1200 J, Stratagene, USA). The membrane was prehybridized in 10 mL hybridization buffer (1x SET, 1% sodium pyrophosphate, 0.6% SDS, 10x Denhard’t, salmon sperm DNA 75 μl) at 65 °C for 2 h, then hybridized with [α-32P]-UTP-labelled probe (10^6 cpm) annealed to the 3′-end approximately nt 600 of BaMV RNA at 65 °C overnight. The membrane was washed with a washing buffer (0.5 × SET, 0.1% SDS, 0.1% sodium pyrophosphate) three times for 20 min each at 65 °C.

**Transient expression in N. benthamiana**

*Agrobacterium* containing NbUbE3R1-OFP, NbUbE3R1/∆TM-OFP, NbUbE3R1/mRING-OFP, OFP or the silencing suppressor HcPro was cultured to OD600 = 1. Five-week-old N. benthamiana OFP, NbUbE3R1/mRING-OFP, OFP or the silencing suppressor Agrobacterium containing NbUbE3R1-OFP, NbUbE3R1/∆TM-OFP or NbUbE3R1/mRING-OFP were further treated with MG132 for 12 h before sample collection. After 3 days post-infiltration, total protein was extracted from inoculated leaves and examined by Western blot analysis.

**Yeast two-hybrid interactions**

The yeast Hybrid Hunter system (Invitrogen, Carlsbad, CA, USA) was used in the study. An interaction between bait and prey protein in the system would activate the expression of the reporter gene (his3 and lacZ) in *Saccharomyces cerevisiae* strain L40. The gene fragment encoding NbUbE3R1/∆TM was constructed into the prey plasmid pYESTrp2 and pLEX, designated pYES-E3/∆TM and pLEX-E3/∆TM. The replication-related DNA fragments were constructed into the bait plasmid pHygLex/Zeo and designated pLEX-Capping, -RdRp and -Helicase. The MPs and CP genes were constructed into the bait plasmid pYESTrp2 and designated pYES-TGBp1, -TGBp2, -TGBp3, and -CP (Huang et al., 2017b). The bait plasmids (pLEX-Capping, -RdRp, or -Helicase) and prey plasmid (pYES-E3/∆TM) or prey plasmids (pYES-TGBp1, -TGBp2, -TGBp3, or -CP) and bait plasmid (pLEX-E3/∆TM) were co-transformed into *S. cerevisiae* strain L40 and selected on Trp /His /Zeo agar plates.

**Bioinformatic analysis**

To inspect gene-specific primers, the sequence from cDNA-AFLP was searched in the *N. benthamiana* sol genome sequence database (http://sydney.edu.au/science/molecular_bioscience/sites/benthamiana/). Web analysis was used to obtain the features of NbUbE3R1. SMART (http://smart.embl-heidelberg.de/) was used to evaluate the content of the protein, and the Aramemnon programme (http://aramemnon.botanik.uni-koeln.de/request.ep) was used to predict the subcellular localization. The sequences were aligned at the Biology Workbench website (http://seqtool.sdsc.edu/).

**Co-immunoprecipitation**

*Agrobacterium* containing NbUbE3R1/∆TM-OFP or OFP alone mixed with the one containing the full length of BaMV infectious cDNA clone containing replicase fused with HA-tag and driven by 35S promoter (pKBR/Rep-HA) in a 1:1 ratio was infiltrated onto N. benthamiana leaves in the presence of HcPro. After 3 days post-infiltration, total protein was extracted from plant leaves by using the extraction buffer containing 20 mM Tris-HCl, pH 7.5, 2 mM MgCl2, 300 mM NaCl, 0.5% NP-40, 5 mM dithiothreitol (DTT) and EDTA-free Protease Inhibitor Cocktail (Roche). The
extracts were centrifuged at 4000 × g at 4 °C for 10 min, and supernatant was subjected to immunoprecipitation by adding 20 µl anti-OFP magnetic beads and rotating with a rotamixer for 4 h at 4 °C. The beads were then washed four times with a binding buffer (20 mM Tris-HCl, pH 7.5, 2 mM MgCl₂, and 300 mM NaCl) and then washed with TBS once before 4x sample buffer was added to release proteins. Finally, samples were subjected to Western blot analysis with anti-HA or anti-OFP antibody.

ACKNOWLEDGEMENTS

We thank the Bioimage Core Laboratory of the Graduate Institute of Biotechnology at National Chung Hsing University for use of the confocal laser scanning microscope. This work was supported by the Ministry of Science and Technology of Taiwan (MOST 107-2313-B-005-043) and (in part) by the Advanced Plant Biotechnology Center from The Featured Areas Research Center Program within the framework of the Higher Education Sprout Project by the Ministry of Education (MOE) in Taiwan.

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**SUPPORTING INFORMATION**

Additional supporting information may be found in the online version of this article at the publisher’s web site:

Fig. S1 Expression profile of ACGT2-1 in BaMV infected *N. benthamiana* verified by semi quantitative Reverse Transcripti on Polymerase Chain Reaction (RT PCR). Mock and BaMV in oculated samples are indicated as M and I, respectively. The samples were harvested at 1, 3, 5 and 7 days post inoculation indicated above each lane. The expression of actin was an internal control.

Fig. S2 Structural features of NbUbE3R1. Illustration of NbUbE3R1 structure: I: low complexity region; II: transmembrane region; III: RING finger domain; and IV: coiled coil region. Fig. S3 Phenotype of control and NbUbE3R1 knockdown plants. Phenotype of *Agrobacterium* mediated knockdown plants at 14 days post infiltration. Phytoene desaturase gene knockdown plants exhibiting a photobleach phenotype were the positive control. Morphology of *NbUbE3R1* knockdown plants did not differ from that of *Luciferase* knockdown plants, the negative control.