MiR398-regulated antioxidants contribute to Bamboo mosaic virus accumulation and symptom manifestation

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Bamboo mosaic virus manipulates reactive oxygen species scavengers to enhance replication and symptom formation.

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Abstract

Virus infections that cause mosaic or mottling in leaves commonly also induce increased levels of reactive oxygen species (ROS). However, how ROS contributes to symptoms is less well documented. Bamboo mosaic virus (BaMV) causes chlorotic mosaic symptoms in both Brachypodium distachyon and Nicotiana benthamiana. The BaMV ∆CPN35 mutant with an N-terminal deletion of its coat protein gene exhibits asymptomatic infection independently of virus titer. Histochemical staining of ROS in mock-, BaMV-, and BaMV ∆CPN35-infected leaves revealed that hydrogen peroxide (H2O2) accumulated solely in BaMV-induced chlorotic spots. Moreover, exogenous H2O2 treatment enhanced yellowish chlorosis in BaMV-infected leaves. Both BaMV and BaMV ∆CPN35 infection could induce the expression of Cu/Zn superoxide dismutase (CSD) antioxidants at messenger RNA and protein level. However, BaMV triggered the abundant accumulation of full-length NbCSD2 preprotein (prNbCSD2, without transit peptide cleavage), whereas BaMV ∆CPN35 induced a truncated prNbCSD2. Confocal microscopy showed that majority of NbCSD2-green fluorescent protein (GFP) predominantly localized in the cytosol upon BaMV infection, but BaMV ∆CPN35 infection tended to cause NbCSD2-GFP to remain in chloroplasts. By 5′-RNA ligase-mediated rapid amplification of cDNA ends, we validated CSDs are the targets of miR398 in vivo. Furthermore, BaMV infection increased the level of miR398, while the level of BaMV titer was regulated positively by miR398 but negatively by CSD2. In contrast, overexpression of cytosolic form NbCSD2, impairing the transport into chloroplasts, greatly enhanced BaMV accumulation. Taken together, our results indicate that induction of miR398 by BaMV infection may facilitate viral titer accumulation, and cytosolic prNbCSD2 induction may contribute to H2O2 accumulation, resulting in the development of BaMV chlorotic symptoms in plants.
Introduction

Plants have evolved sophisticated mechanisms to sense and respond to unfavorable environmental cues. Production of reactive oxygen species (ROS) is a common plant response to various biotic and abiotic stresses. As a very dynamic signaling compartment, chloroplasts can sense biotic and abiotic perturbations to produce pro-defense molecules, including hormones such as salicylic, jasmonic and abscisic acids (ABAs), as well as secondary messengers (calcium and ROS) (Serrano et al., 2016). Typically, virus infection rapidly increases ROS levels (termed an oxidative burst), including hydroxyl radicals (OH), superoxide anions (O₂⁻) and hydrogen peroxide (H₂O₂). SUPEROXIDE DISMUTASES (SODs) are important ROS scavengers in plant cells that convert O₂⁻ to H₂O₂ in the first step of the detoxifying process during pathogen-induced oxidative burst (Desikan et al., 1996), but SOD activity is differentially regulated among diverse virus-infected plants (Riedle-Bauer, 2000; Hakmaoui et al., 2012). Photosystems I and II in chloroplasts are the primary ROS-generating centers, which are rich in oxygen, reductants, and high-energy intermediates (Asada, 2006).

Studies have shown that stress-responsive genes are tightly regulated and fine-tuned by a group of small RNAs (sRNAs), termed microRNAs (miRNAs) (Sunkar et al., 2012). Plant miRNAs are a class of endogenous sRNAs of 20–24 nt that modulate biological and developmental events by negatively regulating gene expression via degradation or translational repression of messenger RNAs (mRNAs) (miRNAs) (Wang et al., 2019). In Arabidopsis thaliana, primary miRNAs with characteristic imperfect stem-loop or hairpin structures are transcribed by RNA polymerase II from MIR genes and then catalyzed by Dicer-Like 1 (DCL1), Hypostatic Leaves 1 (HYL1), Double-Stranded RNA-Binding Protein (DRB) 1/2, and SERRATE (SE) to generate precursor miRNAs (pre-miRNAs). Pre-miRNAs are processed into a 20- to 24-nt miRNA duplex by DCL1, HYL1, and SE, and then methylated at the 3’-terminus by HUA ENHANCER 1, before being exported into the cytoplasm by HASTY 1. In general, miRNA-5p (from the 5’-arm) of the miRNA duplex loads into AGRONAUTA 1 (AGO1) to form an active RNA-induced silencing complex (RISC), which guides RISC to bind target transcripts of complementary sequence. Expression of miRNA target genes is repressed by miRNA cleavage or via a nondegradative mechanism (translational repression) determined by the DCL1 partner proteins, DRB1/2 (Reis et al., 2015).

Virus infection alters the profile of sRNAs by generating virus-derived sRNAs and altering endogenous sRNAs (Zhang et al., 2015), including miRNA accumulation (Mengistu and Tenkegna, 2021). Virus infection can even activate miRNA gene transcription (Bazzini et al., 2009). Constitutive expression of VIRAL SUPPRESSOR OF RNA SILENCING was able to alter miRNA levels and activity (Lewsey et al., 2007; Schott et al., 2012). Apart from miRNAs, a class of virus-activated host-encoded small RNAs (vasiRNAs) may also be induced by RNA virus infection, with some vasiRNAs having been characterized to target genes important in plant immunity (Cao et al., 2014). Studies in animal systems have shown that miRNAs may directly target RNA viruses to restrict (Otsuka et al., 2007; Trobaugh et al., 2014, 2019) or, rather surprisingly, enhance infection (Jopling et al., 2005; Schult et al., 2018). As yet, there is no direct evidence showing direct binding of miRNAs to plant viral genomes, except for some in silico analyses (Satish et al., 2019). However, it has been demonstrated that plant RNA viruses can hijack miRNAs to suppress innate immunity, supporting the notion that viruses manipulate miRNAs to generate a more permissive environment for viral accumulation (He et al., 2008; Varallyay et al., 2010; Li et al., 2012). However, how these virus-responsive miRNAs are regulated has not yet been fully understood.

miR398 is one of the best studied stress-responsive miRNAs and it is evolutionarily well-conserved across the plant kingdom. miR398-3p accumulation is mediated by various abiotic and biotic stresses including salinity, ABA, light, heat, sucrose, copper and iron concentrations, nitrogen deficiency, oxidative stress, and infection by bacteria, fungi, or viruses (Sunkar et al., 2006; Tagami et al., 2007; Dugas and Bartel, 2008; Jagadeeswaran et al., 2009; Jia et al., 2009; Beauclair et al., 2010; Li et al., 2010; Lu et al., 2010; Wu et al., 2011; Liang et al., 2012; Liu et al., 2020). Arabidopsis thaliana possesses three miR398-type miRNAs: miR398a (5p-AGGGUUGAUAUGACACAC and 3p-UGUGUUCUCAGGUCACCUU), miR398b (5p-AGGGUUGAUAUGAGAACACAC and 3p-UGUGUUCUCAGGUCACCUU), and miR398c (5p-AGGGUUGAUAUGAGAACACAC and 3p-UGUGUUCUCAGGUCACCUU) (Sunkar and Zhu, 2004). miR398-3p targets cytosolic (CSD1) and chloroplastic (CSD2) forms of the Cu/Zn SOD, COPPER CHAPERONE FOR SUPEROXIDE DISMUTASE (CCS), MITOCHONDRIAL CYTOCHROME OXIDASE SUBUNIT V (COX5), BLUE COPPER-BINDING PROTEIN (BCBP), and an incompletely annotated plastocyanin-like domain-containing protein, of which the expression is altered according to changing miR398 levels (Sunkar et al., 2006; Beauclair et al., 2010; Li et al., 2010; Brousse et al., 2014). miR398bc regulate CSD2 and CCS via mRNA cleavage and translational repression (Beauclair et al., 2010), but CSD1, COX5, and BCBP expression is regulated by miR398 at the mRNA level (Sunkar et al., 2006; Yamasaki et al., 2007; Brousse et al., 2014). It is also known that miR398bc is transcribed by the SQUAMOSA PROMOTER BINDING PROTEIN-LIKE7 transcription factor under copper-limiting conditions (Sunkar et al., 2006; Dugas and Bartel, 2008; Yamasaki et al., 2009), but CSD2 transcription is unaffected by the presence or absence of copper (Yamasaki et al., 2007). Although multiple studies have shown that miR398 hyper-accumulates in various virus-infected plant species (Tagami et al., 2007; Naqvi et al., 2010; Bazzini et al., 2011; Hu et al., 2011; Pacheco et al., 2012;
Abreu et al., 2014; Suzuki et al., 2019), the biological roles of this group of miRNAs remain unclear. Recently, it was reported that lethal systemic necrosis in potato spindle tuber viroid-infected DCL2/4-knockdown transgenic *Solanum lycopersicum* is accompanied by miR398 upregulation and ROS overproduction (Suzuki et al., 2019). Moreover, increased miR398 accompanied by reduced level of its target umecyanin activates the plant defense responses to restrict beet necrosis yellow vein virus infection in *Nicotiana benthamiana* (Liu et al., 2020).

**Bamboo mosaic virus** (BaMV), a potexvirus of the alphavirus-like superfamily, contains a single-stranded, positive-sense RNA genome with a 5′-mGpppG cap structure and a 3′-poly(A) tail. The viral genome comprises 6,400 nt (excluding the 3′-poly(A) tail), with five conserved open reading frames (ORFs) flanked by 5′- and 3′-untranslated regions (UTRs) of 94 and 142 nt, respectively (Lin et al., 1992, 1994; Yang et al., 1997). ORF 1 encodes a 155-kDa replicase containing three functional domains, i.e. methyltransferase (Li et al., 2001; Huang et al., 2004), helicase (Li et al., 2001), and RdRp (Li et al., 1998) domains. ORFs 2–4 are triple gene block protein (TGBp) genes that encode TGBp1–3, the AtCSD1 and AtCSD2 sequences against the *N. benthamiana* genome and transcriptome database (http://benth genome.qut.edu.au/) (Nakasugi et al., 2013) and found that NbCSD1 (Nbvs.1tr6233164) is cytosolic and NbCSD2 (Nbvs.1tr6229677) is chloroplastic. We designed specific primers to clone full-length cDNAs of NbCSD1 and NbCSD2 by

Results

**H₂O₂ accumulates in BaMV-induced symptomatic tissues**

BaMV infection causes yellowish mosaic and chlorotic symptoms in *N. benthamiana*, with the N-terminal 35 aa of BaMV CP being symptom determinant (Lan et al., 2010). In *Brachypodium distachyon*, BaMV induces brown stripes and some light green spots in inoculated and systemic leaves (SLs) (Liou et al., 2014). In contrast, BaMVΔCP35 infection is symptomless in these two plants (Supplemental Figures S1, B and C). Typically, ROS accumulation and enhanced antioxidant levels accompany symptom development in compatible virus–plant interactions (Song et al., 2009; Rodriguez et al., 2012; Huseynova et al., 2014; Shang et al., 2018). Thus, we wondered if BaMV induces symptoms related to the plants’ ROS response.

We ILs of *N. benthamiana* and *B. distachyon* with BaMV and BaMVΔCP35 virions and then harvested them at 10-d post-inoculation (dpi) when BaMV infection had induced mosaic symptoms. *In situ* histochemical staining of superoxide and H₂O₂ revealed that superoxide distribution was not associated with BaMV-induced symptoms. Superoxide levels were higher in mock-treated relative to BaMV- or BaMVΔCP35-ILs (Figure 1A). However, H₂O₂ only accumulated in symptomatic regions of BaMV- but not BaMVΔCP35-infected IL and SLs of both *N. benthamiana* and *B. distachyon* (Figure 1, B and C; Supplemental Figure S2). Thus, H₂O₂, localized in the yellowish mosaic spots of *N. benthamiana* and in the dark brown stripes of *B. distachyon*. Accordingly, accumulation of H₂O₂, but not superoxide, appears to be restricted to BaMV infection-induced symptomatic tissues.

**NbCSD1 and NbCSD2 are induced at RNA and protein levels by both BaMV and BaMVΔCP35 infection**

There are two possibilities why H₂O₂ accumulates in the chlorotic mosaic spots of BaMV-infected leaves: higher SOD activity or repressed photosynthesis, with this latter resulting in lower superoxide formation during photosynthetic electron transport in BaMV-infected plants compared to that in mock-infected plants. However, superoxide levels in BaMV- and BaMVΔCP35-infected *N. benthamiana* leaves were comparable, despite H₂O₂ levels being undetectable in BaMVΔCP35-ILs by *in situ* histochemical staining (Figure 1, A–C). These results imply that the higher H₂O₂ level in BaMV- than that in mock- or BaMVΔCP35-infected plants may be due to greater SOD activity. SODs can be classified into three groups: CSDs, iron SOD (FeSOD), and manganese SOD (MnSOD), which are localized in cytosol/chloroplasts, peroxisomes/chloroplasts, and mitochondria/peroxisomes, respectively. Superoxides are removed from chloroplasts by FeSOD and CSD (Pilon et al., 2011). An in gel SOD activity assay revealed higher CSD activity in BaMV ILs relative to those in mock and BaMVΔCP35 infection at 10 dpi (Supplemental Figure S3).

To confirm that result, we conducted a BLAST analysis on the AtCSD1 and AtCSD2 sequences against the *N. benthamiana* genome and transcriptome database (http://benth genome.qut.edu.au/) (Nakasugi et al., 2013) and found that NbCSD1 (Nbvs.1tr6233164) is cytosolic and NbCSD2 (Nbvs.1tr6229677) is chloroplastic. We designed specific primers to clone full-length cDNAs of NbCSD1 and NbCSD2 by
reverse transcription PCR (RT-PCR) and conducted 5′-rapid amplification of cDNA ends (RACE) on WT N. benthamiana. We found that the NbCSD1 and AtCSD1 proteins share 70% identity, whereas NbCSD2 and AtCSD2 proteins share 80% identity. Unlike the conserved N-terminal and central regions of NbCSD1 and AtCSD1, the N-terminal transit peptide regions of AtCSD2 and NbCSD2 are hypervariable, yet the SOD motifs of AtCSDs and NbCSDs are highly conserved (Supplemental Figure S4, A–C).

We did not observe any difference in viral CP and TGBp1 levels between BaMV- and BaMVΔCPN35-infected leaves by western blot (Figure 2B). However, RNA and protein levels of NbCSD1 and NbCSD2 were increased in BaMVΔCPN35-infected leaves, and slightly more so in BaMV-infected leaves, relative to mock-infected leaves (Figure 2, A and B). Notably, NbCSD2 was undetectable in mock-infected plants. However, rather than mature NbCSD2 (i.e. lacking transit peptide, ~20 kDa), we detected NbCSD2 preprotein (~31 kDa, prNbCSD2) or a truncated prNbCSD2 (~28 kDa) using anti-CSD2 in BaMV- or BaMVΔCPN35-infected leaves, respectively (Figure 2B). Thus, BaMV and BaMVΔCPN35 infection induces expression of NbCSD1 and NbCSD2 in infected N. benthamiana.
NbCSD2 resides in chloroplasts but targets to cytosol upon BaMV infection

BaMV infection induced accumulation of prNbCSD2 rather than mature NbCSD2 (Figure 2B). We hypothesized that this outcome was due to failed targeting of NbCSD2 into chloroplasts. Therefore, we generated a CSD2- green fluorescent protein (GFP) construct and coinfiltrated it with BaMV or BaMVΔCPN35 to visualize the subcellular localization of NbCSD2. We used CSD2_noTP-GFP harboring mutations at two positively charged residues within the transit peptide as a control (Figure 3A). As expected, in mock-infiltrated cells, CSD2-GFP localized solely in chloroplasts, whereas CSD2_noTP-GFP resided in both cytosol and chloroplasts (Figure 3, A and B). However, in BaMV-infected cells, CSD2-GFP signal mostly emanated from cytosol at the cell periphery and was limited from chloroplasts. In contrast, in BaMVΔCPN35-infected cells, CSD2-GFP signal was mostly associated with chloroplasts and much less so in cytosol (Figure 3B). Thus, we hypothesize that BaMV infection not only induces expression of NbCSD2, but also affects chloroplast targeting of that protein.

H₂O₂ enhances BaMV-induced chlorotic symptoms in N. benthamiana

We already showed that H₂O₂ accumulated in the chlorotic spots of BaMV-infected N. benthamiana leaves but not in symptomless BaMVΔCPN35-infected leaves (Figure 1). Accordingly, we tested if H₂O₂ affects BaMV-induced symptom development by spraying H₂O₂ onto leaves before or after BaMV and BaMVΔCPN35 inoculation. As expected, BaMV infection caused chlorotic spots on N. benthamiana leaves at 10 dpi. However, the BaMV-infected leaves with H₂O₂ treatment either before or after virus inoculation developed more yellowish and bigger chlorotic spots than those without H₂O₂ treatment (Figure 4, A and B). However, no symptoms appeared on the BaMVΔCPN35-ILs, whether or not they had been treated with H₂O₂ (Figure 4A). Moreover, either spraying with H₂O₂ pre- or post-inoculation did not alter virus accumulation in the BaMV- and BaMVΔCPN35-ILs, as revealed by western blot (Figure 4C). Thus, our results reinforce the notion that H₂O₂ could intensify BaMV-induced symptom development without altering BaMV level.

Figure 3 Subcellular localization of CSD2 and CSD2_noTP fusion with GFP (CSD2-GFP and CSD2_noTP-GFP) in mock-, BaMV-, and BaMVΔCPN35-infected N. benthamiana. A, Schematic diagram of CSD2-GFP and CSD2_noTP-GFP. Gray box indicates transit peptide (TP). B, Fluorescent signals of CSD2-GFP and CSD2_noTP-GFP in mock-, BaMV-, and BaMVΔCPN35-infected leaves by confocal microscopic observation. Scale bar indicates 20 μm. Arrowheads indicate localization of GFP signals in chloroplasts. Arrows indicate localization of GFP signals in cytosol. The images of (B) last column were digitally extracted and enlarged for comparison.
NbCSD1 and NbCSD2 are targets of Nb-miR398

CSDs are regulated by a conserved miRNA in plants, miR398, as revealed by 5′-RNA ligase-mediated RACEs (RLM-RACE) of Arabidopsis (Sunkar and Zhu, 2004), by in vivo assay in Oryza sativa (Lu, 2010), and in silico prediction in Pinus (Dugas and Bartel, 2008). Sequence alignment also revealed that a miR398-complementary site exists within the 5′-UTR of NbCSD1 and the coding region of NbCSD2. Therefore, we adapted 5′-RLM-RACE to confirm the presence of Nb-miR398 sites in NbCSD1 and NbCSD2.

Arabidopsis miR398 precursor (pre-miR398) and cDNAs of NbCSD1 or NbCSD2 were cloned into pBin vector for transient overexpression. Leaves of N. benthamiana were then co-infiltrated with Agrobacterium tumefaciens harboring pBin-miR398 and pBin-NbCSD1-cDNA or pBin-NbCSD2-cDNA, before being harvested for RNA purification at 5-d post-agroinfiltration (dpi). By 5′-RLM-RACE PCR followed by sequencing, miR398b-3p cleaved NbCSD1 within the 5′-UTR, whereas it cleaved the NbCSD2 ORF between the 10th and 11th nt of the miR398b-3p complementary site (Figure 5A). Furthermore, we used the Cabbage leaf curl virus (CaLCuV) vector, pCVA-miR398, to transiently overexpress Arabidopsis miR398 precursor in N. benthamiana, enabling us to analyze miR398-mediated regulation of NbCSD1 and NbCSD2 in vivo. In miR398-overexpressing leaves, miR398b level increased 11.4-fold relative to that in vector-control-infiltrated leaves at 5 dpa, whereas NbCSD1 and NbCSD2 levels diminished ~0.65-fold compared to vector control (Figure 5, B and C). Thus, miR398 indeed down-regulates NbCSD1 and NbCSD2 expression in N. benthamiana in vivo via transcript cleavage.

miR398 is induced upon BaMV infection in N. benthamiana

It has been reported previously that levels of miR398-3p change under both biotic and abiotic stresses in Arabidopsis (Zhu et al., 2011), and that expression of miR398-3p and its target genes, CSD1 and CSD2, is important for oxidative stress tolerance (Sunkar et al., 2006). Consequently, to determine if BaMV and BaMV/H17005 CPN35 infection induce accumulation of NbmiR398-3p, we inoculated N. benthamiana with BaMV or BaMV/H17005 CPN35 virions and harvested the ILs at 5 and 10 dpi. Northern blot showed higher levels of NbmiR398-3p in both BaMV- and BaMV/H17005 CPN35-ILs at 10 dpi relative to the mock ILs of WT plants at 10 dpi (Figure 6A). Moreover, levels of NbmiR398-3p were higher in the BaMV ILs than BaMV/H17005 CPN35 ILs (Figure 6A). To monitor how NbmiR398-3p levels were affected by BaMV infection, we analyzed NbmiR398-3p expression in mock- and BaMV ILs at 2-d intervals from 1 to 9 dpi by means of stem-loop RT-qPCR. Our results show that levels of NbmiR398-3p increased progressively with infection duration in both mock- and BaMV ILs. Notably, BaMV infection induced significantly higher NbmiR398-3p levels at 7 dpi, i.e. when mosaic symptoms appear in N. benthamiana (Figure 6B). Thus, mechanical inoculation of BaMV induced NbmiR398-3p upregulation, which coincided with the time-point when disease symptoms are observed in N. benthamiana.

BaMV levels are regulated positively by miR398 and negatively by CSD2

To further investigate the effects of NbmiR398b-3p on BaMV accumulation, we coinfiltrated N. benthamiana with...
Agrobacterium harboring a miR398-overexpression vector (pCVA-miR398) and an infectious clone of BaMV, pKB (Liou et al., 2014). Coinfiltrated leaves were harvested at 5 dpa for RNA purification followed by cDNA synthesis. RT-qPCR revealed that, relative to vector control samples, miR398b-3p levels were increased 60-fold (Supplemental Figure S5A) and BaMV levels were increased 1.84-fold (Figure 7A) in the miR398b-3p-overexpressing N. benthamiana leaves. Consequently, coincident with the high level of NbmiR398b-3p, RNA levels of the NbmiR398b-3p target genes, NbCSD1 and NbCSD2, were decreased in solely pCVA-miR398-infiltrated leaves. However, coinfiltration of pKB with pCVA-miR398 greatly increased NbCSD1 and NbCSD2 gene expression despite NbmiR398b hyperaccumulation (Supplemental Figure S5B). Indeed, NbmiR398b-3p, NbCSD1, and NbCSD2 levels were increased to 2.77-, 3.52-, or 1.76-fold in BaMV-infected N. benthamiana compared to those in vector control-infected leaves, respectively, based on the data from three independent experiments (Supplemental Figure S5, A and B). Moreover, consistent with our results shown in Figure 2B, BaMV infection not only increased accumulation of NbmiR398b-3p but also of the RNAs of its target genes, NbCSD1 and NbCSD2. Thus, NbmiR398b-3p overexpression can enhance BaMV accumulation in N. benthamiana.

We further used short tandem target mimic (STTM) to block miR398 function (Supplemental Figure S6) and analyzed its effect on BaMV levels. N. benthamiana leaves were coinfiltrated with Agrobacterium harboring control pBin61 or pBin-miR398-STTM and Agrobacterium carrying pKB, and then subjected to RNA purification and RT-qPCR analysis at 5 dpa. In miR398-STTM and BaMV co-expressing leaves, BaMV levels were reduced to 0.37-fold relative to that in vector control plus BaMV coexpressing leaves (Figure 7B). Furthermore, in CSD2-silenced plants, BaMV levels had increased 22-fold relative to those in control (phytoene
desaturase (PDS)-silenced) plants (Figure 7C). Taken together, these analyses show that BaMV levels are regulated positively by miR398 and negatively by CSD2.

**NbCSD2 localization affects its regulation role on BaMV level**

We have already shown that BaMV infection causes the failure of CSD2-GFP targeting to chloroplasts (Figure 3B) and CSD2 plays a negatively regulatory role on BaMV level (Figure 6B). We wondered whether CSD_noTP which resides in cytosol has any effects on BaMV accumulation. Therefore, we coinfiltred *N. benthamiana* with *Agrobacteria* harboring a CSD2-HA or CSD2_noTP-HA overexpressed vector and pKB. Coinfilitrated leaves were harvested at 2 dpa for protein purification. By western blot, BaMV CP level was decreased 50% and increased 2.5-fold relative to vector control sample in CSD2-HA and CSD2_noTP-HA overexpressing *N. benthamiana* leaves, respectively, from three independent experiments (Figure 8). This result indicates that BaMV level was regulated negatively by chloroplastic CSD2 and positively by cytosolic CSD2_noTP.

**Discussion**

Pathogen infection often triggers elevated levels of ROS such as superoxides and H$_2$O$_2$, resulting in a hypersensitive response (Lamb and Dixon, 1997). Elevated ROS levels enhance SA content and that of systemic acquired resistance marker proteins such as PATHOGENESIS RELATED 1/2 (Uknes et al., 1992; Maleck and Dietrich, 1999). *Plum plux virus* has been shown to induce chlorotic spots and increased ROS levels in pea plants (Diaz-Vivancos et al., 2008). Moreover, the levels of H$_2$O$_2$, superoxide, and *Cucumber mosaic virus* (CMV)-CP were intercorrelated with symptom development in CMV-infected *N. glutinosa* (Lei et al., 2016; Jing Shang et al., 2018). Similarly, H$_2$O$_2$ was detected in *N. glutinosa* and *N. tabacum cv. Xanthi* infected with the incompatible *Tobacco mosaic virus* (TMV) or *Tomato mosaic tobamovirus* (Madhusudhan et al., 2009). In this study, we show that H$_2$O$_2$ accumulated in BaMV-induced

![Figure 7](image-url)  
**Figure 7** BaMV level was regulated positively by miR398 and negatively by CSD2. Quantification of BaMV by RT-qPCR in (A) miR398 transiently overexpressed plants at 5 dpa, (B) miR398 STTM overexpressed plants at 2 dpa, and (C) CSD2 silenced plants at 7 dpa. Results from three independent experiments, three plants per treatment per experiment, were used for student’s t test. *P < 0.05.
symptomatic tissues of *N. benthamiana* and *B. distachyon*. However, H$_2$O$_2$ was undetectable by 3,3′-diaminobenzidine (DAB) staining of asymptomatic BaMVΔCPN35-infected leaves (Figure 1, B and C). Furthermore, H$_2$O$_2$ treatment enhanced BaMV-induced chlorotic symptoms (Figure 4), but would not cause chlorosis in mock-infected leaves. This may be due to the sprayed H$_2$O$_2$ was not efficient and stable enough for chlorotic induction but it would be a priming signal to induce plant defense response upon pathogen infection. Moreover, levels of catalase that catalyzes H$_2$O$_2$ signal to induce plant defense response upon pathogen infection. Therefore, different pathogens can manipulate miR398 levels to create a better environment for their infection. However, induced miR398 in BNYVV-infected *N. benthamiana* was able to activate immune system to restrict BNYVV infection through silencing a putative miR398 target gene, umecyanin, which is also involved in redox reactions (Liu et al., 2020). In our study, BaMV infection induced miR398 and its target CSD2 accumulation and localization of CSD2 in cytosol. Moreover, BaMV level was increased by overexpressed cytosolic CSD2_noTP-HA but reduced by chloroplastic CSD2-HA protein compared to that of GFP (Figure 8). In addition, BaMV level was also increased in CSD2-silenced plant. Therefore, we hypothesize that BaMV disrupts CSD2 chloroplast targeting to prevent defense signaling system.

We observed increased NbCSD1 and NbCSD2 transcript levels despite hyperaccumulation of miR398 in BaMV-infected *N. benthamiana* leaves (Figure 2), as also found for BaMV-infected miR398-overexpressing *N. benthamiana* (Supplemental Figure S5). These results indicate that BaMV may induce transcription of NbmiR398b-3p, NbCSD1, and 2, B). These data demonstrate that increased H$_2$O$_2$ and antioxidant CSD levels are not associated with BaMV titer, but are linked to BaMV-induced symptoms. In addition, BaMV- but not BaMVΔCPN35 triggered localization of most NbCSD2 in the cytosol (Figure 3). This outcome is likely due to impaired cleavage of the transit peptide of prNbCSD2, so that NbCSD2 could not be properly transported into chloroplasts, resulting in H$_2$O$_2$ accumulation and chlorotic symptoms upon BaMV infection. However, it has previously been shown that, in the case of CMV, the CP of the CMV-M, but not CMV-Q strain, can interact with chloroplastic ferredoxin I (Fd I) protein in the cytosol to disrupt chloroplast transport of Fd I, thereby causing leaf chlorosis (Qu et al., 2018).

CSDs are targets of the conserved miR398 in Arabidopsis (Sunkar and Zhu, 2004) and rice (Dugas and Bartel, 2008; Lu, 2010). By means of 5′-RLM-RACE and in vivo assay, we clearly show that miR398b-3p regulates the NbCSD1 and NbCSD2 genes via transcriptional cleavage in *N. benthamiana* (Figure 5). miR398b-3p and its conserved targets, CSD1 and CSD2, regulate responses to biotic and abiotic stresses, as well as nutrient homeostasis, in many plant species (Zhu et al., 2011). Based on sRNA sequencing data, NbmiR398b-3p is more abundant than NbmiR398b-3p, with this latter being considered the functional miRNA (Yin et al., 2015). However, we showed that NbmiR398b-3p level and BaMV titer increased with infection progression in BaMV-infected *N. benthamiana* by northern blot (Figure 6). Thus, BaMV infection enhances levels of functional NbmiR398b-3p that targets NbCSDs. In addition, BaMV level is positively regulated by miR398 but negatively regulated by CSD2 (Figures 7, A–C and 8). Similar cases have been reported for fungal infection, with transiently overexpressed miR398b from common bean enhancing *Sclerotinia sclerotiorum* fungal lesioning of *N. benthamiana* (Naya et al., 2014). Therefore, different pathogens can manipulate miR398 levels to create a better environment for their infection. However, induced miR398 in BNYVV-infected *N. benthamiana* was able to activate immune system to restrict BNYVV infection through silencing a putative miR398 target gene, umecyanin, which is also involved in redox reactions (Liu et al., 2020).

**Figure 8** Effects of different forms of CSD2 on BaMV accumulation. A, BaMV CP level of BaMV-infected leaves of GFP-, CSD2-HA-, or CSD2_noTP-HA-overexpressed *N. benthamiana* by protein blot at 2 dpa. B, Results from three independent experiments, three plants per treatment per experiment, were quantified by student’s t test. *P < 0.05.
NbCSD2 simultaneously. Several virus-infected plants have been shown to exhibit miR398 accumulation, such as TMV-or Oilseed rape mosaic tobamovirus-infected A. thaliana (Bazzini et al., 2011; Hu et al., 2011), and Potato virus X (PVX)-, Potato virus Y (PVY)-, or Tomato yellow leaf curl China virus (ToLCNV)-infected N. benthamiana (Naqvi et al., 2010; Pacheco et al., 2012). However, PVX and PVY infection resulted in higher NbCSD transcript levels, whereas ToLCNV infection reduced them (Naqvi et al., 2010; Pacheco et al., 2012). Whether or not virus-induced CSD transcript levels are altered may depend on different host-virus combinations.

In uninfected cells, ROS scavengers, CSDs, are tightly regulated by miR398. BaMV infection triggers upregulation of both miR398 and its target CSDs, which miR398 in turn positively regulates the BaMV titer. However, unlike the CSD2 resided in the chloroplasts of uninfected cells, BaMV induced abundant accumulation of PrNbCSD2 retained in the cytosol, leading to H$_2$O$_2$ hyperaccumulation and chlorotic symptoms (Figure 9). In addition, BaMV titer is greatly enhanced by overexpressed cytosolic NbCSD2 but reduced by chloroplastic NbCSD2 (Figure 8). Therefore, we uncover a mediator for BaMV-induced symptom formation and also a regulator of BaMV accumulation.

**Materials and methods**

**Plant growth and BaMV inoculation**

WT N. benthamiana and B. distachyon plants were grown in a growth chamber with 16-h/8-h light/dark cycles at 25°C. For each set of experiments, four 3-week-old N. benthamiana plants at the four-leaf stage were used, and three leaves of each plant were inoculated with virions. For B. distachyon, 7-d-old plants were inoculated with virions. Methods for inoculation were as described previously (Lin and Hsu, 1994; Lin et al., 1996; Liou et al., 2014), except that inocula contained 1 µg BaMV-S or BaMVΔCPN35 virions (Lan et al., 2010).

**Histochemical staining of superoxide and hydrogen peroxide**

Mock-, BaMV-, and BaMVΔCPN35-ILs of N. benthamiana at 10 dpi or of B. distachyon at 7 dpi were detached for histochemical staining as described previously with minor modification (Fryer et al., 2003). Briefly, 0.2% (w/v) Nitrotetrazolium blue chloride (NBT) (Sigma-Aldrich) in 50 mM sodium phosphate buffer (pH 7.5) and 0.2% (w/v) DAB (Sigma-Aldrich) (pH 3.8) were used for superoxide and H$_2$O$_2$ detection. The detached leaves were immediately immersed in freshly prepared NBT or DAB staining buffer followed by vacuum infiltration for 10 min, before being kept in the dark overnight. Then, the leaves were washed once with distilled water and repeatedly shaken in 95% (v/v) alcohol for 20 min until the green color of chlorophyll had disappeared.

**Constructs**

For the NbCSD2 localization assay, CSD2 cDNA was amplified with NbCSD2_XbaI-F and NbCSD2_BamHI-R, and then ligated into XbaI- and BamHI-treated pEpyon-32K (Chen et al., 2011) to generate pEpyon-CSD2-GFP. To construct

![Figure 9](https://example.com/figure9.png) Model of positive regulation of CSDs and H$_2$O$_2$ with BaMV-induced symptoms. In uninfected cell, CSD1 and CSD2 levels are tightly regulated by miR398 and translated into CSD1 and PrCSD2 in cytosol, respectively. PrCSD2 is transported into chloroplasts as CSD2 whose transit peptide is cleaved. In chloroplasts, CSD2 catalyzes the conversion of superoxide (O$_2$-·) into H$_2$O$_2$ which is catalyzed into water by ascorbate peroxidase (APX). After BaMV infection, miR398 and its target CSD1 and CSD2 levels were induced. Moreover, BaMV infection induced PrCSD2 cytosolic retention which may result in H$_2$O$_2$ accumulation and lead to BaMV-induced chlorotic symptoms. Also, miR398 and prCSD2 can enhance BaMV accumulation.
pEpyon-CSD2_noTP-GFP in which NbCSD2 residues K59 and R60 were mutated to A, we used overlap PCR to generate a CSD2_noTP fragment by means of NbCSD2_XbaI-F, NbCSD2-noTP_R, NbCSD2-noTP_F, and NbCSD2_BamHI-R primers (all primers are detailed in Supplemental Table S1). Full cDNAs of BaMV and BaMV△CPN35 were cut from pBS2-8 and pBS△CPN35 (Lan et al., 2010) using XbaI and Sacl, and then ligated into pEpyon-32K treated with XbaI and Sacl enzymes to generate the pEpyon-BaMV and pEpyon-BaMV△CPN35 constructs, respectively. To prepare recombinant CSD1 and CSD2, we amplified the CDS of NbCSD1 and NbCSD2 and inserted them into pET15b before transforming them into Escherichia coli BL21. For protein production, we added 0.1 mM IPTG to E. coli culture at 28°C for 6 h before subjecting it to sonication. Recombinant CSD1 and CSD2 were then purified using His-tag-Ni-NTA resin.

For miR398 overexpression, we used a CalCuV-based miRNA-expressing vector (Tang et al., 2010). The forward primer ath-pmiR398-F with a XbaI site and the reverse primer ath-pmiR398-R with a KpnI site were designed to amplify the miR398 precursor from A. thaliana, which was then ligated into XbaI/KpnI-treated CaLCuV T-DNA pCVA vector. To knock down miR398 expression, we used pCass (Ding et al., 1995) as the template to perform inverted PCR with the primer sets STTM_F_Swal/STTM_R_Swal and 398-STTM F_Swal/398-STTM_R_Swal, respectively. The PCR product was cleaved by Swal and self-ligated to generate pCass-STTM and pCass-398-STTM. Then, we used the plasmids pCass-STTM and pCass-398-STTM as templates to amplify the fragment containing the promoter, STTM sequence and terminator with the primers STTM_F_KpnI and STTM_R_Spel, followed by digestion with KpnI and Spel. The cleaved product was ligated into pBIN61 to generate the clones pBIN61-STTM and pBIN61-miR398-STTM. For CSD2-HA and CSD2_noTP-HA overexpression, pBIN-NbCSD2-cDNA, and pEpyon-CSD2_noTP-GFP were used as template and using the specific primer set NbCSD2_XbaI-F and NbCSD2_HA_BamHI-R for amplification. The PCR product was cloned with XbaI and BamHI before being subjected to PCR clean-up. Vector pEpyon-32K (Chen et al., 2011) was cleaved with XbaI and BamHI, followed by gel purification, and then ligated with the XbaI-NbCSD2_HA_BamHI fragment to generate pEpyon-NbCSD2_HA and pEpyon-NbCSD2_noTP-HA, respectively.

For CSD2 silencing, we amplified the NbCSD2 partial sequence from pBin-NbCSD2-cDNA with the specific primer set NbCSD2_KD_EcoRI and NbCSD2_KD_XhoI. The PCR product was treated with EcoRI and XhoI, subjected to PCR clean-up, and then cloned into pTRV2 (Ratcliffe et al., 2001) that had been cleaved with EcoRI and XhoI, thereby generating pTRV2-CSD2. The NbCSD1 and NbCSD2 cDNAs were cloned into pGEMT-easy vector (Promega, Madison, WI, USA) by using the specific primers listed in Supplemental Table S1 and sequenced. The At-miR398b precursor was cloned into pBin61 vector with primers harboring KpnI or XbaI cutting sites. The pBin-miR398 and pBin-NbCSD1-cDNA or pBin-NbCSD2-cDNA constructs were transformed into A. tumefaciens C58C1 using the freeze-and-thaw method (Weigel and Glazebrook, 2006).

**Agrobacterium infiltration**

All expression vectors were individually introduced into A. tumefaciens C58C1. For NbCSD2 localization, A. tumefaciens harboring pEpyon-CSD2-GFP, pEpyon-CSD2_noTP-GFP, pEpyon-BaMV, or pEpyon-BaMV△CPN35 were cultured overnight and induced with 100 μM acetosyringone in 10 mM MgCl2 to a final optical density at 600 nm (OD600) = 0.2. Agrobacterium containing pEpyon-CSD2-GFP was mixed with that containing pEpyon-BaMV or pEpyon-BaMV△CPN35 in a 1:1 ratio and infiltrated into N. benthamiana leaves, respectively.

For miR398 target site determination, we mixed A. tumefaciens cultures (OD600 = 1) containing pBin-miR398 and pBin-NbCSD1-cDNA or pBin-NbCSD2-cDNA in a 1:1 ratio as indicated, and co-infilitrated them by syringe onto three leaves each of N. benthamiana. At 5 dpa, infiltrated leaves were harvested for RLM-RACE analysis. For the miR398 overexpression assay, A. tumefaciens cultures (OD600 = 0.5) containing pCVA-miR398, pCVB, and pBin61 or pKB (Liou et al., 2014) were mixed in a 1:1:1 ratio and co-infiltrated onto N. benthamiana. At 5 dpa, infiltrated leaves were harvested for RT-qPCR analysis.

For miR398-knockdown assay, A. tumefaciens cultures (OD600 = 0.5) containing pBIN-miR398-STTM or pBIN61 and pKB (Liou et al., 2014) were mixed in a 1:1 ratio and co-infilitrated into N. benthamiana. At 5 dpa, infiltrated leaves were harvested for RT-qPCR. For CSD2 silencing assay, A. tumefaciens cultures (OD600 = 0.5) harboring pTRV1, pTRV2-PDS, or pTRV2-CSD2 were mixed in a 1:1 ratio and co-infilitrated on the first, second, and third leaves of 18-day-old N. benthamiana. At 7 dpa, the fifth and sixth leaves were infiltrated with A. tumefaciens cultures (OD600 = 0.5) containing pBin or pKB. The fifth and sixth leaves were harvested at 5 dpa for further analysis.

**Western blot analysis**

Total protein was extracted (Vijaya Palani et al., 2006), separated by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, electrotransferred onto PVDF membranes (Immobilon-P; Millipore), and then detected with rabbit anti-NbCSD1, -CSD2, -BaMV-TGBp1 (Chang et al., 1997), and -CP (Lin and Chen, 1991) antisera at 1:5,000 followed by probing with horseradish peroxidase-conjugated goat anti-rabbit antibody (Abomics, New Taipei City, Taiwan) at 1:10,000. NbCSD1- and NbCSD2-specific antisera were prepared by Abomics by immunizing rabbits with NbCSD1-specific synthetic polypeptides (N-DAPPTVTVNSGLKPGFHG and NGTAPFTITDKQVPLAGPSI) conjugated to the C-terminal of carrier protein key-hole limpet hemocyanin (KLH) and NbCSD2-specific synthetic peptides (N-TSSTNSSLFPVAAP
Expression of BaMV and the putative miR398 target genes CSD1 and CSD2 was assayed by RT-qPCR. Two micrograms of total RNA was reverse-transcribed using a ToolsQuant II Fast RT Kit (Biotools, New Taipei City, Taiwan). The qPCR reactions were carried out in triplicate with SYBR-Green I Core Reagents (Life Technologies, Carlsbad, CA, USA) in a GeneAmp® 9700 Sequence Detection System (Life Technologies, Carlsbad, CA, USA) programmed to hold at 50°C for 2 min, then run at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Specific primers for BaMV-RdRp, CSD1, CSD2, and actin (as internal control) are listed in Supplemental Table S2 for PCR reactions. PCR results reflected change in fluorescence signal of SYBR-Green I dye, and the Ct value for each reaction was determined in ABI Prism 7000 SDS software (Life Technologies, Carlsbad, CA, USA) setting the threshold of fluorescence as the exponential phase of amplifications.

**Supplemental Data**

The following materials are available in the online version of this article.

- **Supplemental Figure S1.** Alignment of CSD1 and CSD2 from Arabidopsis thaliana and N. benthamiana.
- **Supplemental Figure S2.** Expression of miR398, NbcSD1 and NbcSD2 in miR398 overexpressed leaves of N. benthamiana leaves with or without BaMV infection harvested at 5 d post-agroinfiltration by RT-qPCR.
- **Supplemental Figure S3.** Higher CSD activity in BaMV-infected leaves.
- **Supplemental Figure S4.** Alignment of CSD1 and CSD2 from Arabidopsis thaliana and N. benthamiana.
- **Supplemental Figure S5.** Expression of miR398, NbcSD1 and NbcSD2 in miR398 overexpressed N. benthamiana leaves with or without BaMV infection harvested at 5 d post-agroinfiltration by RT-qPCR.
- **Supplemental Figure S6.** Expression of NbcSD2 in miR398-STTM overexpressed leaves of N. benthamiana harvested at 5 d post-agroinfiltration by RT-qPCR.
- **Supplemental Figure S7.** Catalase level was not altered by BaMV and BaMVΔCPN35 infection in N. benthamiana.
- **Supplemental Figure S8.** Silencing of catalase induced chlorotic-like symptoms in N. benthamiana.
- **Supplemental Table S1.** Primer lists for cloning.
- **Supplemental Table S2.** Primer lists for RT-qPCR.

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**Conflict of interest statement.** The authors declare that there is no conflict of interest.
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