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The role of miRNAs in plant development and virus defense

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三等獎

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作者簡介

我是童宇鴻，目前就讀建國中學二年級。我從小就對科學有極大的熱忱，不只喜歡觀察生活周遭的事物，更愛仔細尋找萬事萬物背後所隱含的深刻道理。我喜歡閱讀，並從中了解作者嘔心瀝血得到的成果，體會他們的心路歷程，品味其思想的精華，進而增進自己的學養，豐富自己的生活。

作科展讓我初步體會到研究生活的辛苦，使我脫離過去對科學天真爛漫的幻想，在實驗過程中也遇到許多困難。我慢慢了解，作研究不同於學校考試，不只需要對知識的理解與記憶，更需要嚴謹的邏輯推理，以及解決問題的能力，更重要的，認真嚴肅的態度。科展拓展了我的視野，使我進步，讓我茁壯。

微型 RNA 在植物生長發育及對抗病毒中所扮演的角色

摘要：

微型 RNA 是最近發現的小 RNA，調控生物體內的反應，包括生長、細胞分化、對抗病毒…等。植物利用 RNA 干擾 (RNAi) 或過敏反應 (HR) 對抗病毒感染。有趣的是，miR168 可藉由降解 mRNA 或抑制轉譯，調控阿拉伯芥 AGO1 的表達，而 AGO1 是 RNAi 的一個重要元件。miR398 則調控銅鋅超氧化物歧化酶 (CSD1, CSD2) 的表達，而 CSD1, CSD2 負責產生過氧化氫去引發細胞凋亡 (cell apoptosis)。帶有竹嵌紋病毒 (BaMV) 全長基因的轉殖菸草 (*Nicotiana benthamiana*) 品系 27-17 是我們的研究材料。27-17 的幼葉不具病徵，隨著葉子的生長，病徵會漸漸變嚴重。我發現被病毒感染時，植物會提高 AGO1 的表達，使 RNAi 更有效率。然而，病毒藉提高 miR168 使 AGO1 的量無法上升。植物亦可提高 CSD1, 2 mRNA 的量，促進細胞凋亡。病毒卻會引發 miR398 降解 CSD2 mRNA。在病毒力價高的葉子中，雖然 CSD2 mRNA 降低且 miR398 升高，植物仍可大量提高 CSD2 蛋白的量。CSD1 mRNA 沒有被 miR398 負調控，詳細原因仍有待研究。

The role of miRNAs in plant development and virus defense

Summary:

miRNAs are a group of newly discovered small RNA which control a vast array of biological process, including growth, cell differentiation, virus defense, to name a few. When infected by virus, plants exploit RNA interference (RNAi) and hypersensitive response (HR) as defense. Interestingly, the expression of an important protein involved in RNAi, *ARGONAUTE 1* (*AGO1*), is regulated by miR168 in *Arabidopsis* through both mRNA cleavage as well as translational repression. In *Arabidopsis*, miR398 down-regulates *Cu/Zn superoxide dismutase 1* & *2* (*CSD1* & *2*), which are responsible for producing hydrogen peroxide to activate cell apoptosis program, depriving virus of replication materials and confining it to a small region, which is an important part of HR. However, the detailed mechanism and regulatory roles of these two miRNAs in virus defense are still unclear. A transgenic line of *Nicotiana benthamiana*, 27-17, which contains full-length genome of Bamboo mosaic virus (BaMV), is used as the research material. Interestingly, the mosaic and necrotic symptoms are progressively developed with the leaf maturity, e.g. only fully-expanded leaves exhibit severe symptoms. I utilized qRT-PCR to measure gene expression levels, and Western blot for protein detection. In my study, AGO1 mRNA is up-regulated in the presence of virus. Nonetheless, virus could induce miR168 and suppress AGO1, giving rise to lower efficiency of RNAi. Plants also increase the transcriptional rate of CSD1 and CSD2 in order to promote cell apoptosis, but miR398 is triggered and suppress CSD2 mRNA, contributing to more efficient infection. As a response, plants could increase CSD2 protein under the circumstances of high-level miR398 and low expression of CSD2 mRNA. CSD1 mRNA doesn't seem to be down-regulated by miR398 in *N.*

benthamiana. The actual reason is still under investigation.

Contents

Introduction:

The regulatory roles of miRNAs in organisms are now a newly emerging topic. miRNAs can regulate gene expression via either mRNA cleavage, translational repression or DNA methylation (Baulcombe, 2004). The former two mechanisms are called post-transcriptional gene silencing, and they are extremely critical for plant development (Zhang *et al.*, 2006). miRNAs are a class of non-coding endogenous small RNAs, consisting of about 20-24 nt. Every miRNA precursor has a stem-loop hairpin structure, and it will further be processed by Dicer-like (DCL) proteins to form a miRNA: miRNA* duplex (Filipowicz *et al.*, 2005). The duplex will be transported to cytoplasm by HASTY, and miRNA will be loaded into an ARGONAUTE protein (AGO1) to form RNA-induced silencing complex (RISC), whereas miRNA* is degraded (Mallory & Vaucheret, 2006). mRNAs with sequences complementary to the miRNA will then either be cleaved or be reduced in translational rate. As a result, this fine-tuned mechanism keeps gene expression in control.

An extensively similar process in plants exists, called RNA interference (RNAi). When plants are infected by RNA virus, the endogenous or virus-encoded RNA-dependant RNA polymerase (RdRp) can convert viral RNA into double-stranded RNA (Mallory & Vaucheret., 2006). This dsRNA will then be processed into siRNA by DCL proteins, and the siRNA is loaded into AGO1 to form RISC and induces cleavage of viral genomic RNA (Voinnet, 2001).

In both process, AGO1 exhibits one of the most important components. Jones-Rhoades and Bartel (2004) identified some plant miRNAs and their target genes by computational analysis, and AGO1 homeostasis is reached by the coordination of miR168-programmed *AGO1* mRNA cleavage and AGO1-mediated stabilization of

miR168 (Vaucheret *et al.*, 2004; Vaucheret *et al.*, 2006; Vaucheret, 2009). In addition, Varallyay *et al.* (2010) showed that miR168 is induced and AGO1 is suppressed in *Nicotiana benthamiana* infected with *Cymbidium ringspot virus* (CymRSV) and other distinct viruses. Therefore, how this feedback mechanism reacts to the infection of RNA virus is an interesting topic.

In addition to RNAi, hypersensitive response (HR) is another means for plants to fight against RNA viruses (Fig. 1). Oxidative burst is an important part of hypersensitive response. Reactive oxygen species (ROS) are produced, elementarily containing H_2O_2, OH・, O_2^-, etc. O_2^- could undergo a reaction catalyzed by superoxide dismutases (SODs)

$$2\ O_2^- + 2\ H^+ \rightarrow H_2O_2 + O_2$$

The reaction could converts O_2^- into H_2O_2 (Herouart *et al.*, 1993; Mehdy, 1994), and H_2O_2 is able to trigger both local and systemic defense responses and activate cell apoptosis (Tenhaken *et al.*, 1994). Hence, virus is deprived of replication materials and confined to a small region filled with dead cell.

It has already been proved by 5'-RACE and computational methods that miR398 targets to CSD1, CSD2 in *A. thaliana* (Jones-Rhoades & Bartel, 2004). miR398 was found to be down-regulated under biotic and abiotic stress (Sunkar *et al.*, 2006; Jagadeeswaran *et al.*, 2009), followed by *CSD1* and *CSD2* increment in order to convert O_2^- into H_2O_2, which is less poisonous. As a result, plants could protect cells from damage caused by ROS. To our knowledge, there's no research focusing on the link between virus infection and miR398 so far. Thus, we decided to conduct research on the unknown interaction.

Bamboo mosaic virus (BaMV) is a plant virus of alphavirus superfamily with a genome of positive single-stranded RNA. The genome encodes RNA replicase, three overlapping movement proteins and coat protein (Fig. 2). Since BaMV has been the limiting factor for the bamboo industry in Taiwan (Lin *et al.*, 1979), the molecular biology has been substantially studied. Most importantly, the full-genome of BaMV cDNA clones is available.

Recently, a transgenic *N. benthamiana* line 27-17 expressing full-length BaMV genome has been generated. In line 27-17, only fully-expanded leaves exhibit serious mosaic symptoms, however, young and immature leaves are asymptomatic (Fig. 3). This provides us with a material to investigate the interaction between plant and virus, for we can acquire cells with different virus titer from each leaf. In this study, we investigated whether miR168 and miR398 modulate BaMV accumulation and symptom development in BaMV transgenic *N. benthamiana* line.

Methods and processes:

Plant material

25-day-old *N. benthamiana* wild type (WT) and BaMV transgenic line 27-17 are grown in growth chamber, with 14 hr illumination at 28 ^{o}C and 10 hr darkness at 25 ^{o}C each day (Fig. 3A). We give each leaf a score according to symptom severity, and observe eight plants of line 27-17 (Fig. 4). Counting from base to tip, except cotyledon, the fifth (fully-expanded, L5) leaf exhibits severe symptom while sixth (L6) leaf has slight symptom, and seventh (young, L7) leaf does not have visible symptom. Thus, they were harvested for further experiments (Fig. 3B).

Total RNA extraction

Total RNA was isolated from L5, L6 and L7 of *N. benthamina* using TRIzol (Invitrogen), and then the quality as well as concentration of RNA was analyzed by Nanodrop Spectrophotometer ND-1000 (J&H Technology) and 1% agarose gel electrophoresis.

qRT-PCR for miRNA

The relative expression levels of miR168 and miR398 were measured in different leaves of line 27-17 and WT by qRT-PCR (Varkonyi-Gasic & Hellens, 2010). Total RNA (1 μg) is reverse transcribed by **stem-loop RT primers** (Fig. 5) and SuperScript™ III Reverse Transcriptase kit (invitrogen). The RT reaction was first maintained at 50 ^{o}C for 1 hr and stopped at 85 ^{o}C for 5 min.

qPCR was carried out using POWER SYBR GREEN PCR MASTER MIX (Applied Biosystems Inc.) in an ABI prism 7500 (Applied Biosystems Inc.), with 25 ng cDNA and gene-specific forward primers and universal reverse primer (URP) (Table 1). U6 snRNA was used as reference gene. The reaction was preceded with 40 cycles of 95 ^{o}C (15 sec) and 60 ^{o}C (1 min). The 95 ^{o}C step stands for denaturing, while the 60 ^{o}C step stands for annealing and extending.

qRT-PCR for mRNA of AGO1, CSD1 and CSD2

Sequences of *AGO1*, *CSD1* and *CSD2* in *N. benthamiana* are not available yet, so information from NCBI Genbank is used to acquire sequences of *Arabidopsis thaliana* and other plant species. The software MegAlign 7.1 (DNASTAR Inc., CA., USA) is used for multiple sequences alignment, looking for the most conserved region to design the primers (Fig. 6 and 7). To have a mutual optimal annealing temperature, it is

important that the lengths and GC pair numbers of the forward and reverse primer should be close. Additionally, our primers are usually located at exon-intron border for the purpose of eliminating the false positive resulting from genomic DNA contamination.

RT-PCR, followed by gel electrophoresis, was carried out previous to qRT-PCR so as to examine the PCR quality. After several tests of RT-PCR with different annealing temperature, different cycles as well as different primers, we've eventually acquired partial length of our wanted genes. The resulting PCR product was sequenced and compared with genomes of other plant species with BLAST. The similarity between our sequences and genes from other species were assessed to determine whether the sequences belong to our goal genes. The similarity between our putative CSD1 and *A. thaliana* CSD1 and CSD2 is 70% and 60%, respectively. The similarity between our putative CSD2 and A. thaliana CSD1 and CSD2 is about 60% and 70%. We then redesigned our primers according to the newly acquired sequences.

Protein extraction

First, 0.1 g leaves were grinded with liquid nitrogen. After that, 100 μl 2X protein buffer (Table 2) was added and boiled for 5 min. Then samples were centrifugated at 13,000 rpm for 30 min, 4^{o}C and the supernatant, which contains total proteins, was extracted.

To concentrate the protein sample, 4-fold volume of acetone was added and completely mixed. Samples were stored at -70 ^{o}C deep freezer. After centrifugation and 70% EtOH washing, samples were re-suspended in 2X protein buffer.

Western blot

Polyclonal antibodies against AGO1, CSD2 were obtained from Agrisera Inc. Total proteins were separated by SDS-PAGE using NuPAGE 4-12% Bis-Tris Mini Gels (invitrogen) and were transferred to PVDF membrane in CAPS buffer (pH =11.1). Western Lightning^{TM} Plus-ECL chemiluminiscence reagent (PerkinElmer Inc., Netherland) was utilized for signal detection.

Results and Discussions:

More severe symptoms in leaves are associated with more coat protein of virus

Total protein of BaMV transgenic *Nicotiana benthamiana* line 27-17 was extracted for Western blot analysis of BaMV coat protein (CP). Coat protein expression level is higher in symptomatic L5 than asymptomatic L7 in 27-17. That is to say, the amount of coat protein is positively correlated with symptom severity (Fig. 8).

Induction of miR168 is observed in leaves with more viruses

Using the BaMV transgenic line 27-17, we investigated the relationship between miR168 and virus titer. The 5th leaves (L5) to 7th leaves (L7) of *N. benthamiana* WT and transgenic line 27-17 were harvested and total RNA was extracted for assays. miR168 expression level is similar in all ages of leaves in WT; however, it is positively correlated with virus titer in 27-17 (Fig. 9A). miR168 is substantially up-regulated in symptomatic leaf (L5) of 27-17, suggesting virus induction of miR168.

The mRNA expression of AGO1 gene is negatively correlated with that of miR168, but higher in 27-17 than in WT

First of all, *AGO1* mRNA level is higher in young leaves (L7) than in old leaves

(L5) in both WT and 27-17 (Fig. 9B), indicating that young leaves (L7) require more AGO1 for many physiological functions, and the higher expression of AGO1 probably could account for lower virus titer in young leaves. However, *AGO1* mRNA expression isn't decreased in line 27-17, which is contradictory to the fact that miR168 level is higher in 27-17 than in WT. Why? Recall that AGO1 protein is an important component of RISC, which plays one of the most important roles in viral RNA interference. Thus, it is reasonable that *AGO1* mRNA increases when plants are infected by RNA virus. Nevertheless, virus could circumvent part of the destruction caused by RNAi by way of triggering the expression of miR168 in plants (Fig. 10).

The expression of miR398 is higher in leaves with more virus titer

We investigated the expression level of miR398 in 27-17 and WT. miR398 is up-regulated significantly in L5 of BaMV transgenic line 27-17. In addition, miR398 expression remains constant in all ages of leaves in WT, but is positively correlated with virus titer in 27-17 (Fig. 11A). The results lead to the conclusion that more virus exists can induce more miR398 expression, and this is in accordance with many recently reported data. Diermann *et al.* (2010) reported that in tomato plants inoculated with potato spindle tuber viroid (PSTVd), miR398 is induced about 3.6 folds according to Solexa sequencing data. Naqvi *et al.* (2010) also used microarray to investigate miRNA expression profile in tomatoes infected by tomato leaf curl new delhi virus (ToLCNDV), and they found out that miR398 is up-regulated after infection. However, they didn't further investigate why miR398 is up-regulated and the expression pattern of target genes of miR398.

According to some previous studies, however, miR398 is suppressed and *CSD1*, *CSD2* is up-regulated when plants are under some abiotic (Sunkar *et al.*, 2006;

Yamasaki *et al.*, 2008; Jagadeeswaran *et al.*, 2009) or biotic stress (Jagadeeswaran *et al.*, 2009), which are associated with oxidative burst, and CSD1, CSD2 can convert O_2^- into H_2O_2, which is less poisonous, and H_2O_2 could be further degraded by catalase, protecting plants from the damage caused by ROS.

miR398 is suppressed when plants are under some abiotic stress, but why is miR398 induced under viral infection? For further investigation, we tested CSD1, CSD2 mRNA.

The expression of CSD1 mRNA is positively correlated with both miR398 and virus titer

In our study, CSD1 mRNA level is similar in all ages of leaves in WT, but it is induced in 27-17 L5, in which miR398 is up-regulated and virus titer is high (Fig. 11B). It has already been confirmed that miR398 targets to CSD1 and CSD2 by 5'-RACE (rapid amplification of cDNA ends) in *A. thaliana* (Jones-Rhoades & Bartel, 2004). Is it reasonable that more miR398 is accompanied by more *CSD1* mRNA? Our data suggests that CSD1 is not a target gene of miR398 in *N. benthamiana*, or the silencing pathway is blocked (Fig. 12).

CSD2 mRNA is induced by plants but suppressed by miR398

In our study, *CSD2* mRNA level remains the same in different ages of leaves in WT (Fig. 13A). Compared with WT, CSD2 mRNA is up-regulated in young leaf (L7) but down-regulated in old leaf (L5) of 27-17. Moreover, the expression level of CSD2 mRNA is negatively correlated with miR398 in 27-17, suggesting CSD2 mRNA cleavage by miR398.

We could infer that plants increase the expression level of CSD2 mRNA,

accounting for the up-regulation of CSD2 in asymptomatic L7 of 27-17. However, high-titer virus in 27-17 L5 induces miR398, suppressing CSD2 mRNA, and the net effect is the decrease in CSD2 mRNA expression.

The protein level of CSD2

Since translational repression is an alternative way for miRNAs to regulate their target genes (Lanet *et al.*, 2009; Beauclair *et al.*, 2010; Varallyay *et al.*, 2010), the expression level of CSD2 protein in WT and 27-17 was measured by Western blot. Younger leaves (L7) express more CSD2 protein in WT. However, CSD2 protein is highly expressed in 27-17 L5, though miR398 is increased and CSD2 mRNA is reduced (Fig. 13B). The result is quite confusing because miR398 may down-regulate either CSD2 protein level or CSD2 protein and mRNA both. We could infer that plant utilizes an unknown pathway to increase CSD2 protein level in leaves with high virus titer (27-17 L5), and three strategies are possible: increase CSD2 translational efficiency, increase CSD2 mRNA lifespan, or increase CSD2 protein lifespan.

More ROS leads to more CSD2 protein production, leading to more conversion of O_2^- into H_2O_2. H_2O_2 could drive the cross-linking of cell wall structural proteins and lead to cell apoptosis (Tenhaken *et al.*, 1994). In other words, H_2O_2 could help trap viruses in a region in which cell death could deprive viruses of replication materials. However, virus up-regulates miR398 and suppresses CSD2, in order to decrease H_2O_2 and cell apoptosis. CSD2 mRNA is thus decreased, but CSD2 protein is instead increased. In terms of the fact that CSD2 protein mainly exists in chloroplasts, we inferred that plants transport more CSD2 protein to chloroplasts, protecting CSD2 from degradation in chloroplasts. Therefore, CSD2 protein could accumulate to high amount though mRNA level is low and miR398 is abundant (Fig. 14).

Future plans

To validate our conclusions, more experiment is needed. Next, we will exploit *Agrobacterium* infiltration (Cazzonelli and Velten, 2006) to over-express miR168-resistant AGO1 in 27-17. If symptom is relieved, virus titer drops and siRNA increases, it may support our hypothesis that AGO1 is really the limiting factor for plants to combat RNA virus. Virus-induced gene silencing (Lu *et al.*, 2003) of pre-miR168 is the next step. If virus titer drops and AGO1 mRNA increases, we could state that virus up-regulates miR168 in order to reduce AGO1, thus inhibiting RNAi.

5'-RACE will be performed to confirm whether CSD1 and CSD2 are the targets of miR398 in *N. benthamiana*. In our study, CSD1 mRNA doesn't seem to be the target of miR398. Similar to miR168 and AGO1, over-expression of miR398-resistant CSD2 and suppression of pre-miR398 is needed for validation of the regulatory role of miR398 in symptom formation. In addition, we will also treat 27-17 with H_2O_2 scavenger, in an attempt to determine the probability of hypothesis advocating that symptom formation is part of a result of H_2O_2 mediated cell apoptosis.

After determining the role of miR168 and miR398 in plant virus defense, other genes will be tested in DNA microarray and miRNA microarray in order to further investigate other genes involved in BaMV infection.

Conclusions and applications:

Plants combat virus infection by gene silencing. Thus, virus can reach high accumulation level after initial infection but decline to low titer soon due to plant recovery. In contrast, transgenic *N. benthamiana* expressing full-length cDNA of BaMV, line 27-17, with progressive symptom formation and virus titer, provides us with a steady material for studying the insight mechanism of how miRNAs are involved in virus infection and plant defense.

After long time of evolution, plants and viruses have both developed complicated and fine-tuned mechanism to combat each other. When infected by virus, plants usually employ RNA interference and hypersensitive response as defense against pathogens. Our study revealed that plants will raise AGO1 mRNA expression level in order to increase RNAi. However, virus could trigger miR168 to down-regulate AGO1 through mRNA cleavage. This anti-defense method leads to a new concept that miRNAs are involved in virus defense and symptom formation.

Hypersensitive response causes oxidative burst, producing a lot of reactive oxygen species (ROS). Plants exploit enzymatic and non-enzymatic mechanisms to remove those ROS. The former includes superoxide dismutases (SODs), catalases and peroxidases etc. SODs are mainly composed of Cu/Zn SOD (CSD1, CSD2), Fe SOD and Mn SOD. Research on how these enzymes are regulated is of fundamental importance. In this study, we found out that miR398 and *CSD1* is up-regulated in leaves with higher virus titer. Unlike in *Arabidopsis thaliana*, miR398 doesn't seem to target CSD1 in *N. benthamiana*, but it's clear that CSD1 mRNA is highly expressed in symptomatic leaf (L5) of 27-17 to promote cell death. We also found out that *CSD2* is triggered in leaves with low virus titer but suppressed in those with higher virus titer. It

is surprising since previous studies discovered that plants repress miR398 and thus induce *CSD1* and *CSD2* in order to remove ROS under abiotic stress or infected by pathogens (Jagadeeswaran *et al.*, 2009). It struck to us that it might be BaMV virus that increases miR398 expression, leading to CSD2 mRNA down-regulation.

Despite low molecular concentration of *CSD2* mRNA and high level of miR398, CSD2 protein is still up-regulated in leaves with high virus titer (27-17 L5), and the mechanism is still under investigation. However, we could conclude that the homeostasis of CSD2 protein in 27-17 may be due to virus repression and plant induction under biotic stress.

Our research really disclosed the mysterious, amazing defense, counter-defense interaction between plants and viruses, from the viewpoints of both RNAi and hypersensitive response, with miRNAs playing a vital role. The pathway we discovered is illustrated in Fig. 15.

The story doesn’t stop here.

Although more experiments are clearly needed, our data hint a role of miRNAs in RNAi regulation and symptom development after plant virus infection. Global analysis of differential expression of miRNAs should lay the ground work of how miRNAs are involved in defense and counter-defense mechanism between plants and virus. We are continuing exploring the amazing world of plants and viruses, dedicated to revealing the rules lying behind our beautiful Mother Nature.

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Appendix:

Table 1. Sequences of miRNAs and primers in qRT-PCR for miRNA and mRNA

The red parts in primers represent the sequences complementary to miRNAs

	Sequence 5'→3'	Purpose
miR168	UCGCUUGGUGCAGGUCGGGAA	
miR168-F	GCGGCGGTCGCTTGGTGCAGGT	miR168a forward primer for qPCR
miR168-RT	GTTGGCTCTGGTGCAGGGTCCGAGGTATT CGCACCAGAGCCAACTTCCCG	Stem-loop reverse primer of miR168a for RT
miR398	UGUGUUCUCAGGUCGCCCCUG	
miR398-F	GCGGCGGTGTGTTCTCAGGTCG	miR398b forward primer for qPCR
miR398-RT	GTTGGCTCTGGTGCAGGGTCCGAGGTATT CGCACCAGAGCCAACCAGGGG	Stem-loop reverse primer of miR398b for RT
URP	GTGCAGGTCCGAGGT	Universal reverse primer in qRT-PCR for miRNA
U6-F	GGACATCCGATAAAATTGGAACGATACAG A	U6 serves as reference gene in qRT-PCR for miRNA
U6-R	AATTTGGACCATTTCTCGATTTATGCGTGT	
AGO1-F	CTGGTGGCATGATAAAGGAATTGCTTAT	
AGO1-R	CCTTGCGGATTGCATCAAGTTCA	
CSD1-F	TGCCCTTGGTGATACCACAAATGG	
CSD1-R	GCAAGAGGAATCTGCTTGTCAGTAATGG	
CSD2-F	CAAGGGAAATTCCAATGTTGAGGG	
CSD2-R	GTTTGTAGTGTCACCGTACTCGTGTAAATG	
Nb-actin-F	TACGAGCTGCCTGATGGACAA	*N. benthamiana actin* is reference gene in qRT-PCR for mRNA
Nb-actin-R	GCTTCCATTCCGATCATTGATG	

Table 2. Amount and purpose of each ingredient of 2X protein buffer

	Amount (mL)	Purpose
1 M Tris (pH 7.4)	2.5	Maintain pH
10% SDS	10	Denature native proteins to individual polypeptides
100% glycerol	10	Maintain the solution under low temperature without freezing
β-ME	0.9	Break disulfide bond
ddH_2O	27.5	
Total	50	

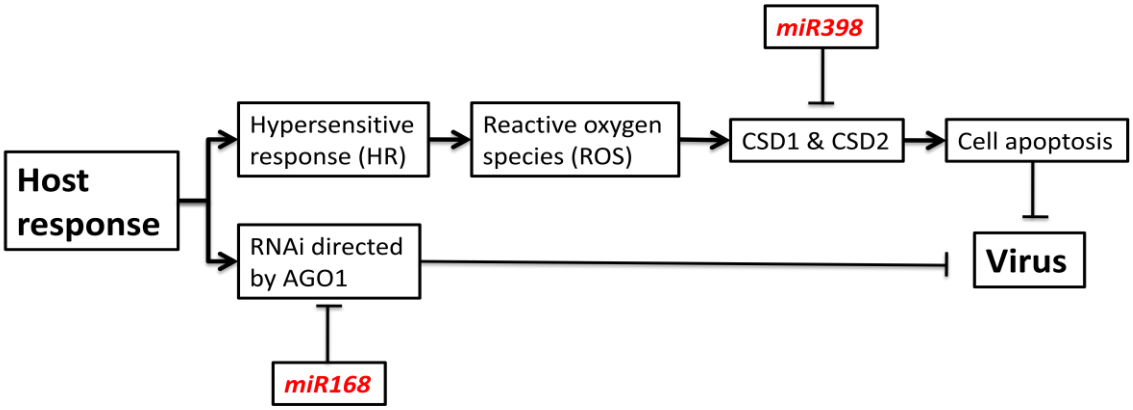
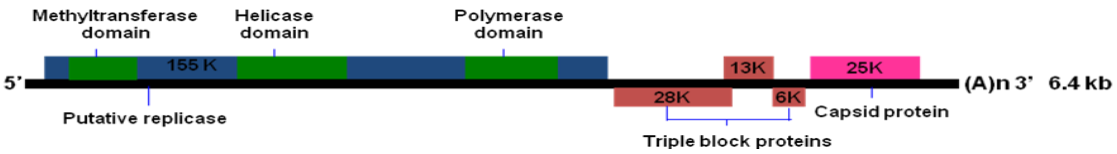


Fig. 1. Plant defense mechanism includes hypersensitive response (HR) and RNAi

(A)



(B) (C)

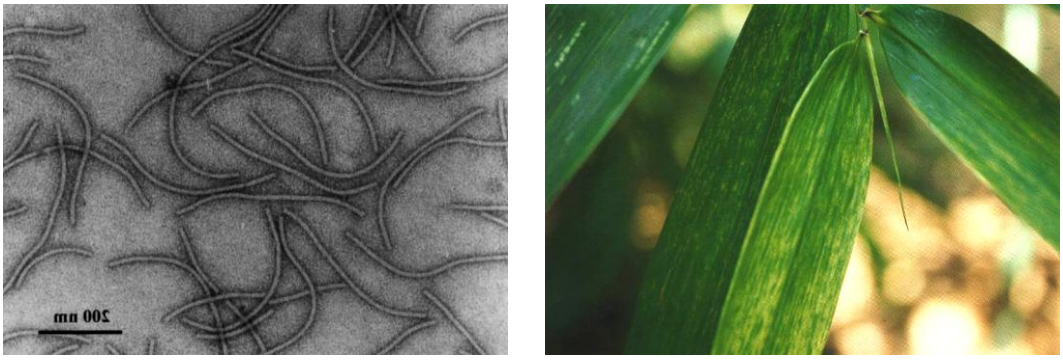


Fig. 2. Bamboo mosaic virus (BaMV) genome organization (A), morphology (B) and BaMV infected bamboo leaves (C)

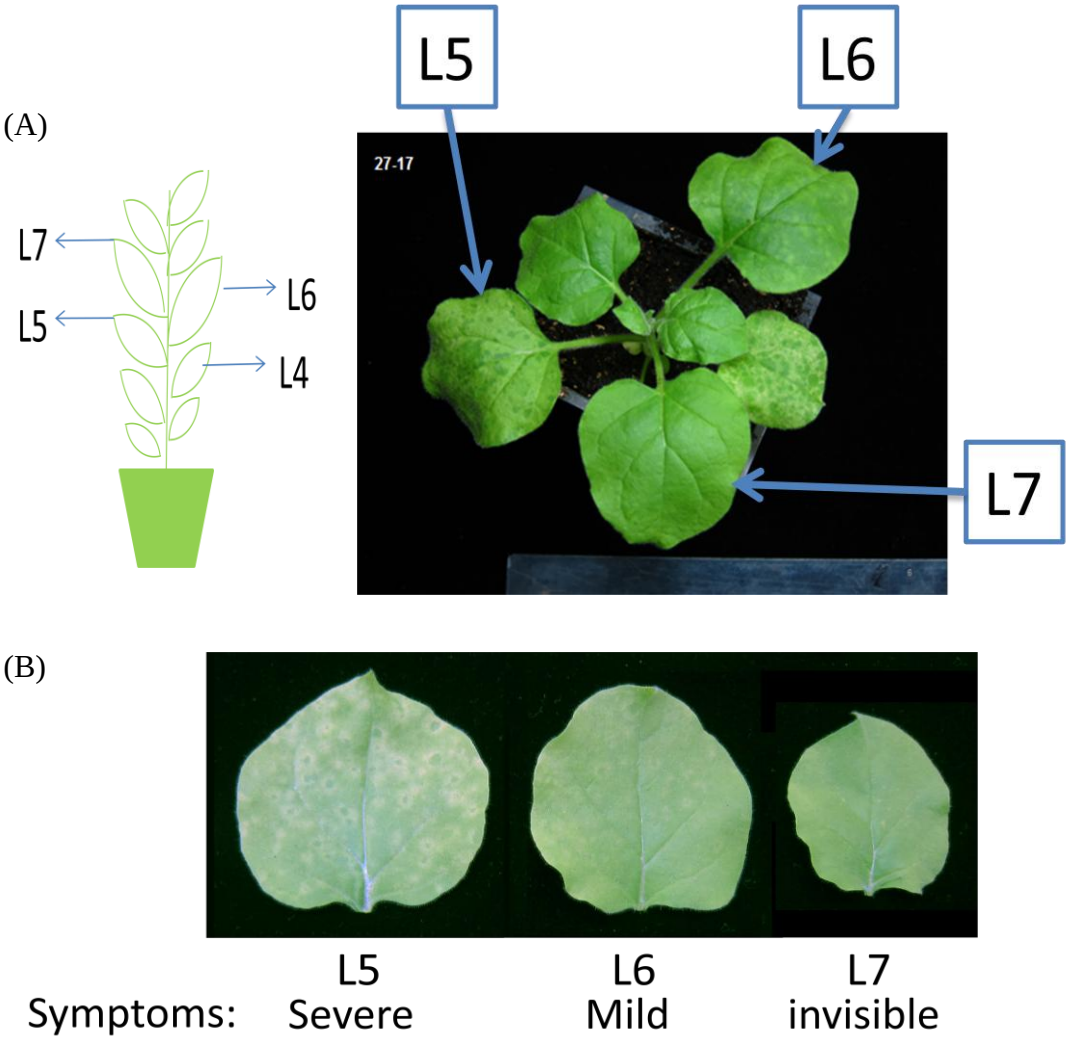
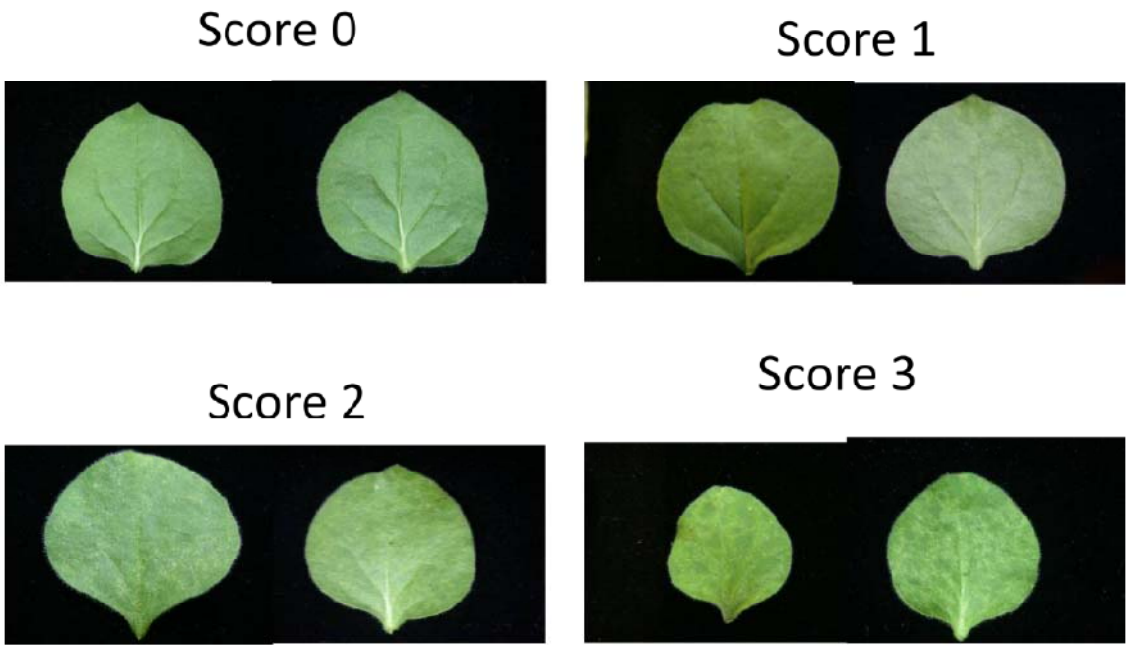


Fig. 3. Phenotype of BaMV-transgenic line 27-17

(A) A transgenic line 27-17 plant. L4 to L7 represents 4th to 7th leaves

(B) Each leaf of 27-17 exhibiting different level of symptom: the symptom of L5 is obvious, while that of L6 or L7 is subtle

(A)



(B)

plant / Leaves	Plant 1	Plant 2	Plant 3	Plant 4	Plant 5	Plant 6	Plant 7	Plant 8	average
L4	2	3	3	2	2	2	2	2	2.25
L5	1	1	1	0	1	1	1	1	0.88
L6	0	0	0	0	0	0	0	0	0
L7	0	0	0	0	0	0	0	0	0

Fig. 4. Symptom severity

(A) The score scale of symptom severity

(B) Scores in different-stage leaves of line 27-17

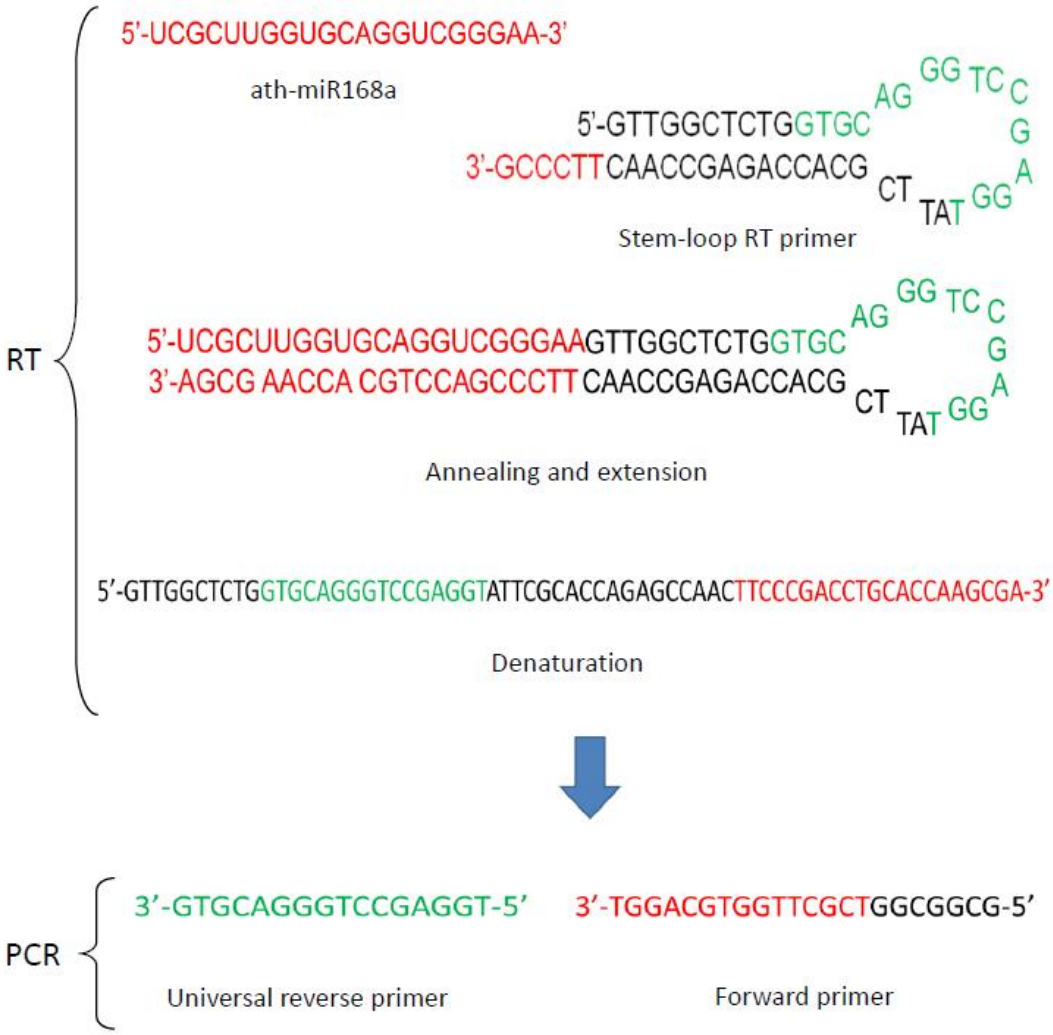


Fig. 5. Scheme of miRNA qRT-PCR

Stem-loop reverse transcription primer for ath-miR168a. The red part represents the sequence complementary to ath-miR168a, and the green part stands for the sequence of universal reverse primer.

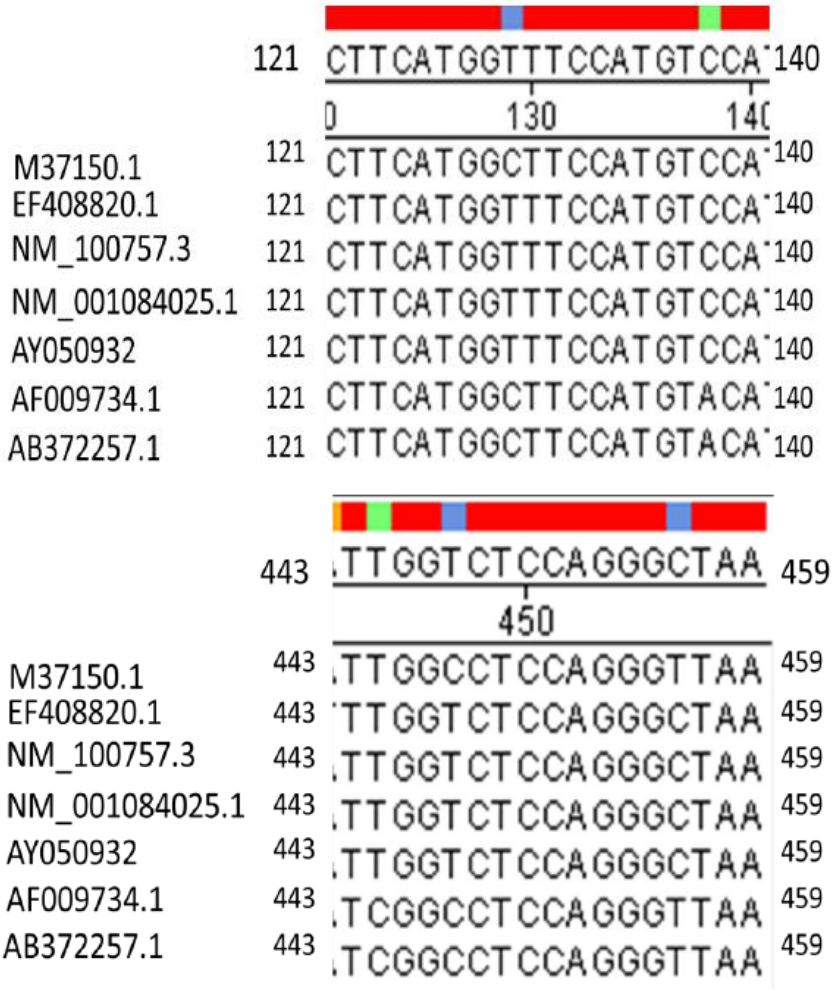


Fig. 6. Sequence alignment of *CSD1* by MegAlign 7.1 (DNASTAR Inc.)

1. F-primer: 5’-CTTCATGG(C/T)TTCCATGT(C/A)CA -3’
2. R-primer: 5’-TTA(A/G)CCCTGGAG(A/G)CC(A/G)A -3’
3. Predicated length of PCR product: 338 nt

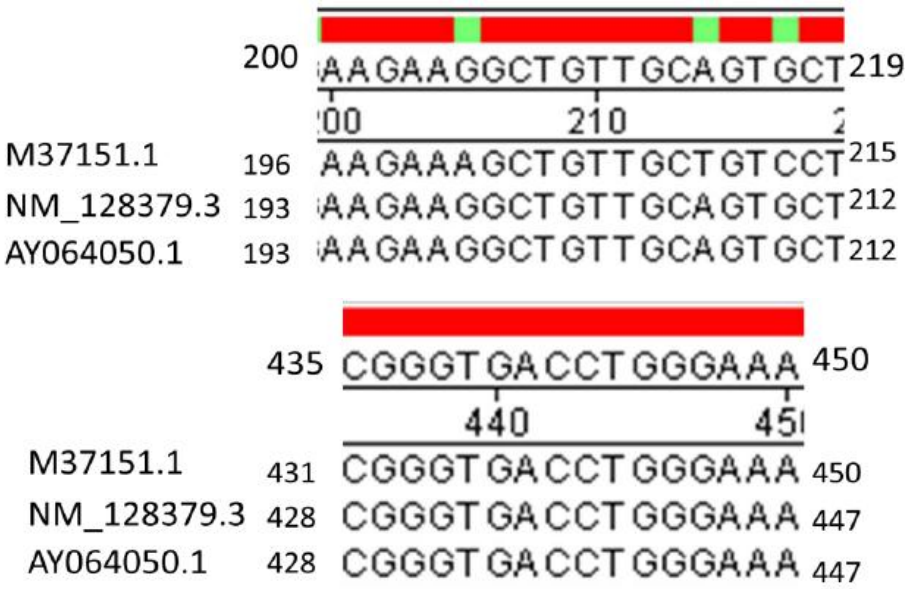


Fig. 7. Sequence alignment of *CSD2* by MegAlign 7.1 (DNASTAR Inc.)

1. F-primer: 5’-AAGAA(A/G)GCTGTTGC(A/T)GT(G/C)CT -3’
2. R-primer: 5’-TATGTTTCCCAGGTCACCCG -3’
3. Predicated length of PCR product: 250 nt

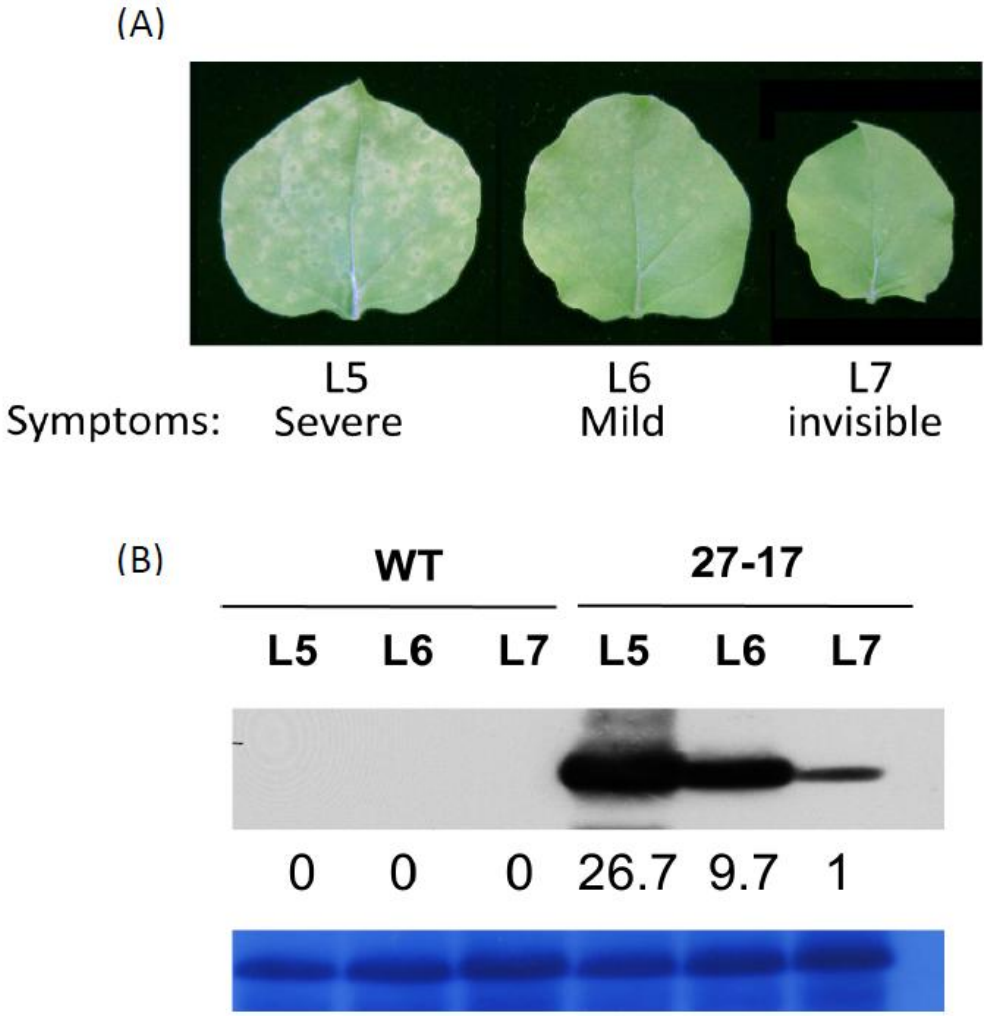
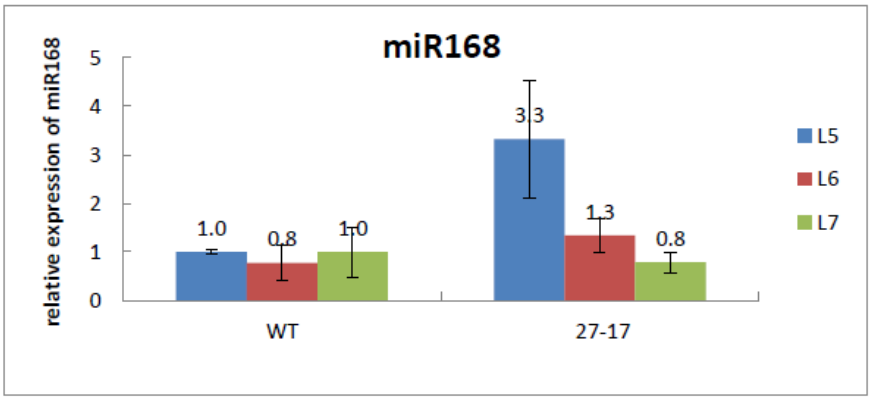


Fig. 8.

(A) The symptom severity in different ages of leaves from 27-17

(B) Western blot shows the relative concentration of BaMV coat protein (CP) in different leaves. 27-17 L5 > L6 > L7. CP amount is positively correlated with symptom severity

(A)



(B)

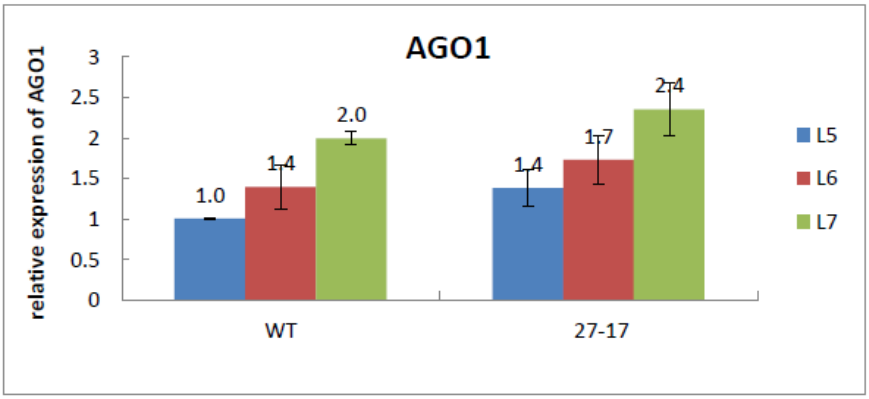
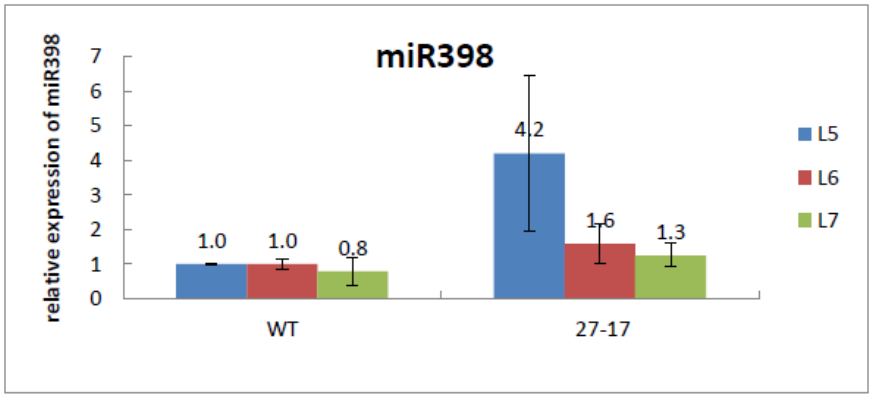


Fig. 9. The relative expression levels of miR168 (A) and AGO1 mRNA (B) in BaMV transgenic line 27-17 and WT are measured by qRT-PCR.
The relative expression in WT L5 is set as 1.0. U6 and actin are used as reference gene for miR168 and AGO1, respectively. Error bars indicate standard deviation (miR168, n=3; AGO1 mRNA, n=4)

Fig. 10. During virus infection, plant increases AGO1 mRNA. However, virus triggers miR168 to suppress AGO1 mRNA. Consequently, the net effect is the similar AGO1 mRNA expression level in 27-17 and WT.

(A)



(B)

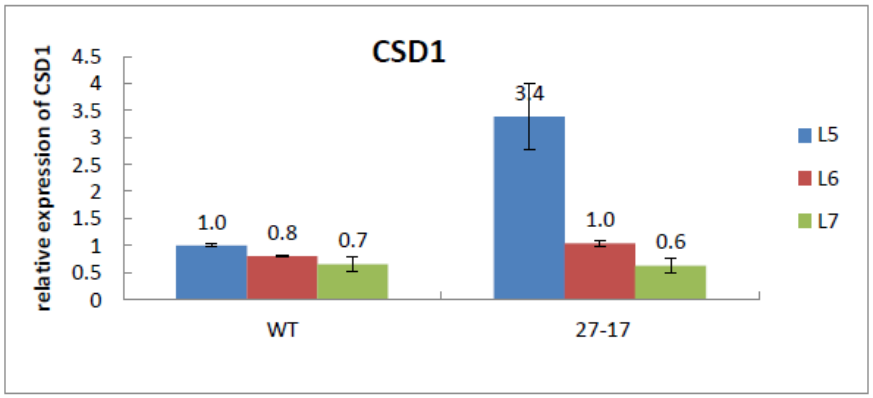


Fig. 11. The relative expression levels of miR398 (A) and CSD1 mRNA (B) in BaMV transgenic line 27-17 and WT are measured by qRT-PCR.
The relative expression in WT L5 is set as 1.0. U6 and *actin* are used as reference gene for miR398 and CSD1, respectively. Error bars indicate standard deviation (miR398, n=3; CSD1 mRNA, n=4)

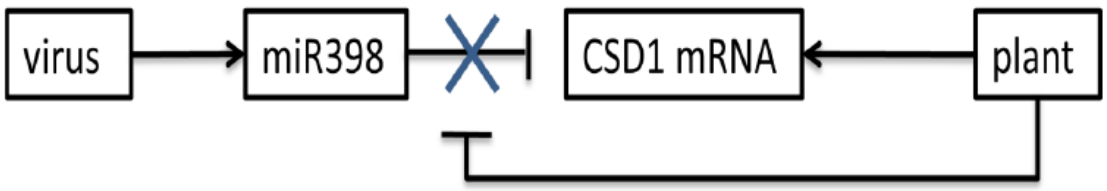


Fig. 12. When infected by virus, plant induces CSD1 mRNA, and virus increases miR398 as response, but the gene silencing pathway of miR398 to CSD1 is blocked or doesn't exist in *N. benthamiana*

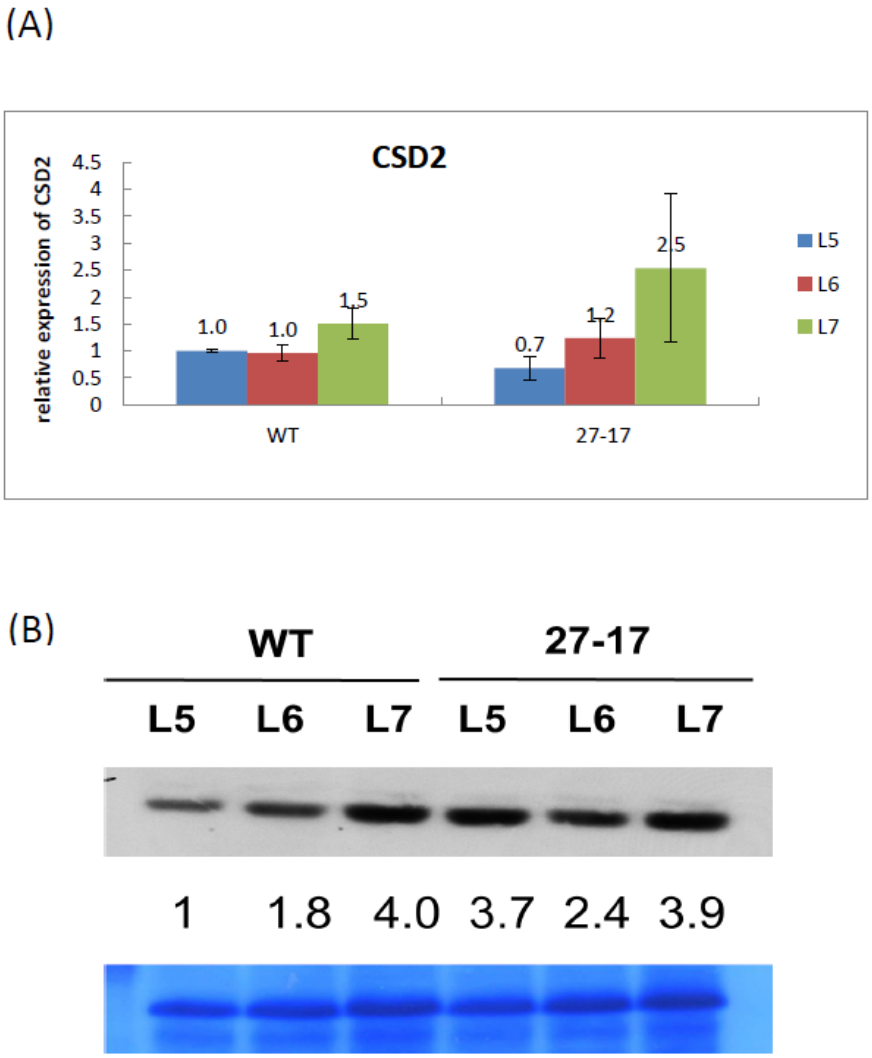


Fig. 13.

(A) CSD2 mRNA expression levels are measured by qRT-PCR. The relative expression in WT L5 is set as 1.0. actin is used as reference gene for CSD2. Error bars indicate standard deviation (n=4)

(B) CSD2 protein is detected by Western blot. Coomassie blue staining of total protein is used as loading control

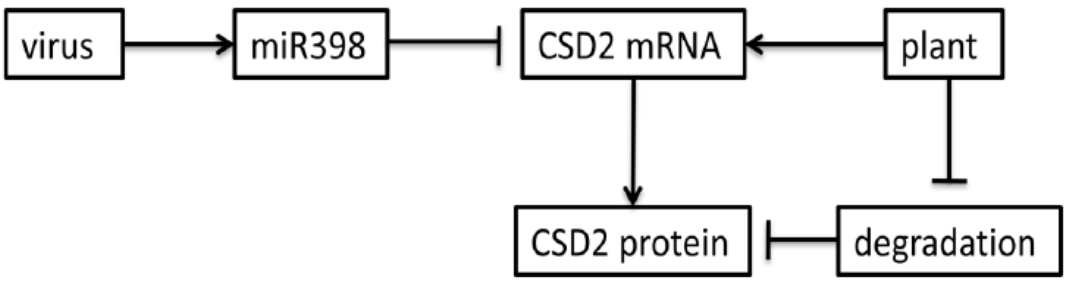


Fig. 14. Plant induces CSD2 mRNA to eliminate ROS. High-titer virus induces miR398, suppressing CSD2 mRNA expression. When virus reaches high amount, CSD2 protein lifespan may be prolonged

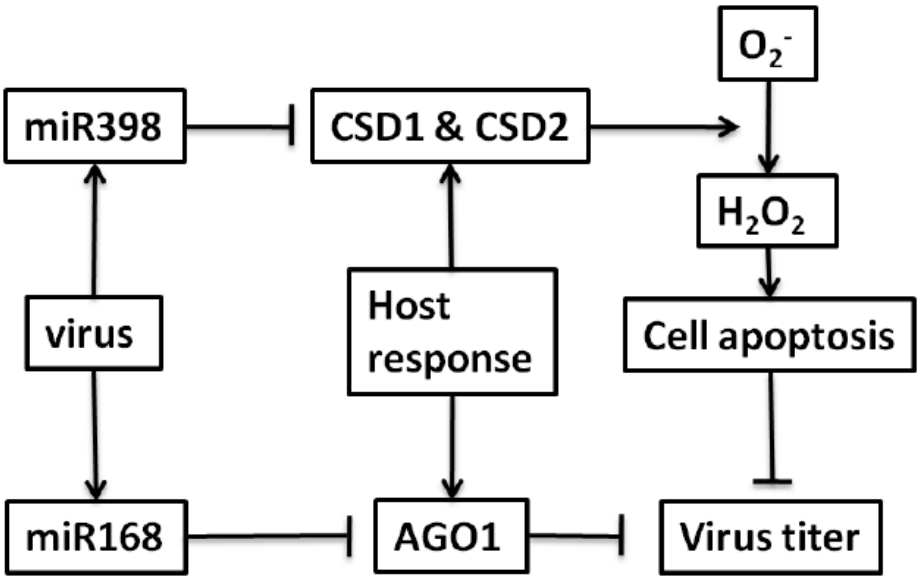


Fig. 15. A speculative pathway of interaction between plants and virus, in which miR168 and miR398 plays a crucial role.

評語

探討微型 RNA 在植物發育過程及防禦機制中扮演的角色。具學術研究及運用價值的潛力。研究過程按部就班有系統，並有初步的結論。然仍需更進一步的探討，以確定研究目的的答案。