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A novel function for a ubiquitous plant enzyme pectin methylesterase: The enhancer of RNA silencing

Yuri L. Dorokhov[a], Olga Y. Frolova[a], Eugene V. Skurat[a], Peter A. Ivanov[a], Tatjana V. Gasanova[a], Anna A. Sheveleva[a], Nikolay V. Ravin[b], Kristiina M. Mäkinen[c], Victor I. Klimyuk[d], Konstantin G. Skryabin[b], Yuri Y. Gleba[d], Joseph G. Atabekov[a,*]

[a] *Department of Virology and A.N. Belozersky Institute of Physico-Chemical Biology, Moscow State University, Leninsky Gory 1, Laboratory Building A, Moscow 119992, Russia*
[b] *Center "Bioengineering" Russian Academy of Science, Moscow, Russia*
[c] *Department of Applied Biology, University of Helsinki, Finland*
[d] *Icon Genetics GmbH, Biozentrum Halle, Germany*

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Abstract Co-agroinjection of *Nicotiana benthamiana* leaves with the pectin methylesterase (proPME) gene and the TMV:GFP vector resulted in a stimulation of virus-induced RNA silencing (inhibition of GFP production, virus RNA degradation, stimulation of siRNAs production). Conversely, co-expression of TMV:GFP with either antisense PME construct or with enzymatically inactive proPME restored synthesis of viral RNA. Furthermore, expression of proPME enhanced the GFP transgene-induced gene silencing accompanied by relocation of the DCL1 protein from nucleus to the cytoplasm and activation of siRNAs and miRNAs production. It was hypothesized that DCL1 relocated to the cytoplasm may use as substrates both miRNA precursor and viral RNA. The capacity for enhancing the RNA silencing is a novel function for the polyfunctional PME.

Keywords: Pectin methylesterase; Tobacco mosaic virus; RNA silencing

1. Introduction

Posttranscriptional gene silencing (PTGS) or RNA interference (RNAi) is a sequence-specific gene-regulatory mechanism including virus-induced gene silencing (VIGS) and transgene-induced gene silencing (TIGS). The core mechanisms of RNAi are shared among different eukaryotes (for review, see [1–5]). The molecular mechanism of RNAi includes: (i) the processing of double-stranded (ds) RNAs into small (21–25 nucleotides) RNA molecules (siRNAs); (ii) incorporation of one of the two strands of the siRNA into the so-called RNA-induced silencing complex (RISC) for subsequent targeting and degradation of mRNAs with complementary target sequence. RNase III-like enzymes termed dicer (DCR) [6,7] and ARGONAUTE [8] proteins are involved in RNAi. DCR cleaves dsRNA or stem-loop RNA into siRNAs. Several types of cellular DCR-like (DCL) enzymes are involved in production in plants of: (i) miRNA (DCL1) [9,10]; (ii) viral siRNA (DCL2) [11]; (iii) endogenous siRNA, responsible for chromatin modification (DCL3) [10,11]; and ta-siRNA (DCL4) [12,13]. The plant viruses are strong inducers of RNAi, while most of them induce a counter-defense reaction by expressing the protein suppressors of RNAi [14].

Identification of the host proteins involved in the RNAi would be helpful for better understanding of this phenomenon. Pectin methylesterase (PME) catalyses the deesterification of pectin, participates in modulating of cell wall (CW) during plant growth and morphogenesis [15]. It has been reported that tobacco PME binds the movement protein (MP) of *Tobacco mosaic virus* (TMV) [16,17], suggesting that PME is involved in the cell-to-cell movement of plant viruses. In higher plants the PME genes encode N-terminal extension (pro-sequence, PS) of different length upstream of the mature PME. We found that tobacco proPME gene encodes the 255-aa N-terminal PS including 24-aa transmembrane (TM) domain; deletions in TM region abolished the delivery of PS:GFP fusion into the CW [18].

Here, we showed that PME is an efficient enhancer of RNAi. The transient expression of proPME gene in *Nicotiana benthamiana* leaves resulted in a significant enhancement of RNAi induced by TMV. We also demonstrated that PME was involved in TIGS. This effect of PME was accompanied by relocation of DCL1 from nucleus to cytoplasm and activation of siRNA and miRNA production.

2. Materials and methods

2.1. Plasmids used for agroinjection

Viral vector crTMV:GFP and binary vectors expressing the full-length PME gene and its derivatives were described earlier [19]. The binary-based plasmid, NtGUT1, encoding tobacco CW pectin glucuronyltransferase was kindly provided by Dr. S. Satoh. *A. tumefaciens* with plasmids encoding miR171prec, DCL1:GFP and DCL2:GFP were kindly provided by Dr. T. Okuno, Dr. M. Matzke and Dr. J. Carrington, respectively.

2.2. Agroinjection procedure

A. tumefaciens strain GV3110 was grown at 28 °C in L-broth supplemented with 50 μg ml^{-1} rifampicin and 25 μg ml^{-1} gentamicin to the stationary phase. Bacteria were separated by centrifugation at $5000 \times g$ for 5 min at room temperature and resuspended in 10 mM $MgSO_4$, MES, pH 5.6. Cells were left in this medium for 3 h at room

*Corresponding author. Fax: +7 495 938 0601.
E-mail address: atabekov@genebee.msu.su (J.G. Atabekov).

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temperature and then injected into the abaxial air spaces of 6-week-old *N. benthamiana* plants. Transient co-expression with the constructs described above was carried out with bacterial suspensions at OD_{600} of 0.6.

2.3. siRNA and miRNA isolation and Northern blot analysis

Isolation of low molecular weight RNA and Northern analysis was executed as described by [20,21].

2.4. GFP imaging

The GFP fluorescence was monitored by illumination with a handheld UV source (DESAGA). A dissecting microscope (Opton IIIRS), coupled to an epifluorescence module, was used for single cell observations. Lower epidermal cells of injected leaves were analyzed at 72 h after agroinjection. Confocal imaging was performed using an inverted Carl Zeiss LSM 510 laser scanning microscope (Jena).

3. Results

3.1. Inhibition of TMV:GFP virus reproduction upon co-agroinjection with proPME gene

In plants infected with TMV:GFP virus, the efficiency of GFP expression correlates with the level of virus reproduction and RNA accumulation [22–24]. *N. benthamiana* leaves were

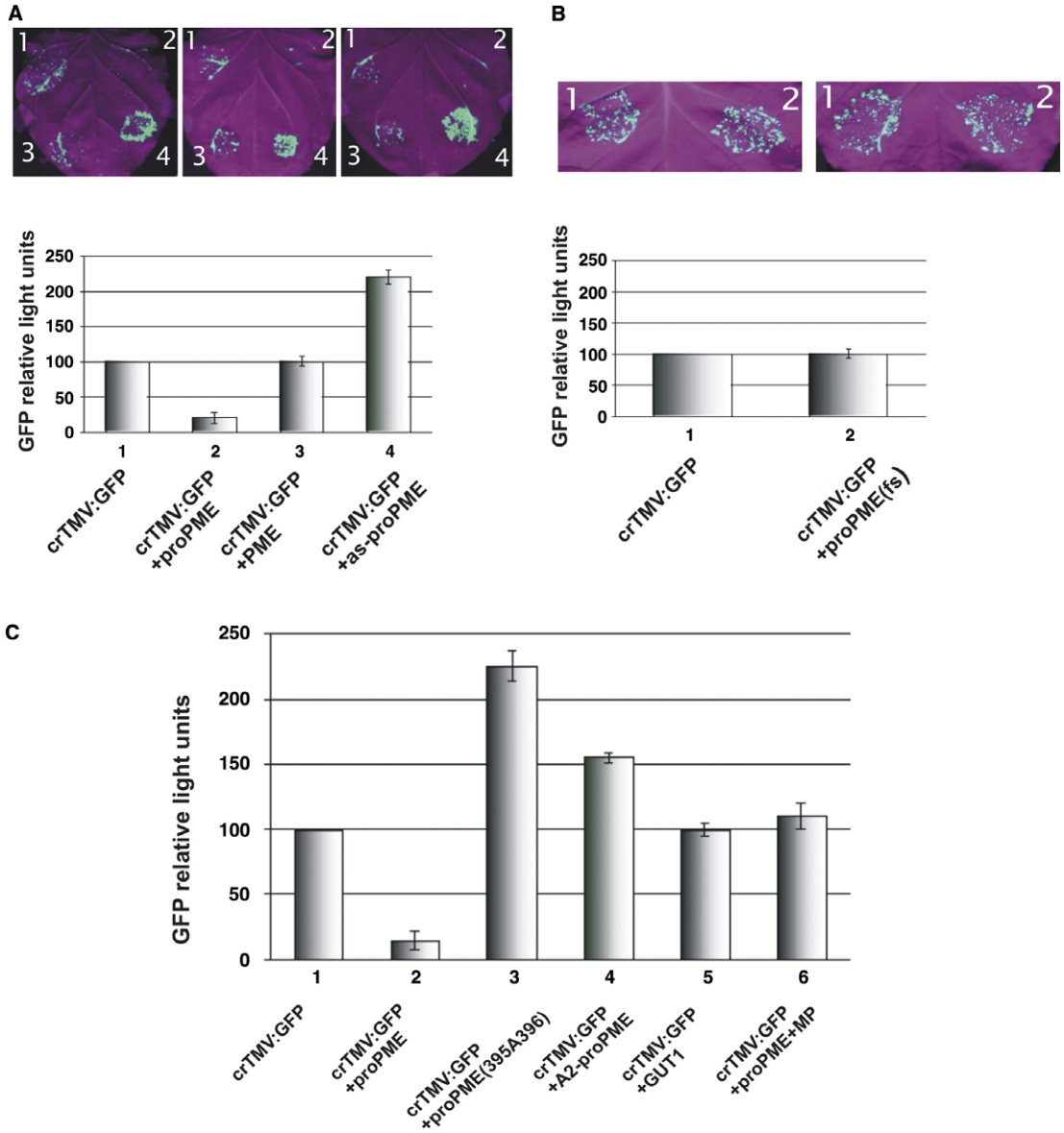

Fig. 1. Dependence of TMV:GFP reproduction on proPME gene expression. (A) Agroinjected GFP-expressing leaf spots under UV illumination (upper part) and fluorimetric analysis of GFP expression (bottom part). Three individual leaves of *N. benthamiana* are presented. Four parts of leaves were agroinjected with (1) crTMV:GFP virus vector alone, or with: (2) proPME gene, (3) mature PME gene and (4) as-proPME construct. Equal concentration of specific *Agrobacteria* in injection samples was maintained by dilution with *Agrobacteria* containing empty vector. Leaves were harvested at five days after injection and GFP expression was measured by fluorimetric analysis. The data obtained were corrected for auto fluorescence. The units shown are based on an arbitrary scale. In each experiment, GFP fluorescence induced by crTMV:GFP vector was taken for 100 relative light units. The mean values (with S.E. bars) for 5–8 independent experiments are given. (B) Two opposite parts of leaves were agroinjected with: crTMV:GFP (1) or crTMV:GFP + proPME(fs) mixture (2). (C) Co-expression of crTMV:GFP with PME mutants and NpGUT1; TMV MP abolished the proPME-mediated inhibition of crTMV:GFP expression.

coagroinjected with binary vectors containing: (i) proPME gene or its derivatives; and (ii) crTMV:GFP vector virus with the CP gene substituted by GFP. The vector was based on the genome of a tobamovirus infecting cruciferous plants (crTMV) [19]. An important advantage of agroinjection procedure is that it allows infecting as much as 94% cells of the inoculated leaf [23], ensuring co-expression of crTMV:GFP and PME in one cell.

The crTMV:GFP virus vector induced visible spots of GFP expression (Fig. 1A1). Remarkably, joint agroinjection of crTMV:GFP and proPME resulted in a suppression of GFPexpressing spots development (Fig. 1A2). Measurements of GFP fluorescence confirmed that co-expression of proPME caused more than fourfold inhibition of virus-mediated GFP production (Fig. 1A2, bottom, and C2). Contrary to the 70-kDa full-length proPME, the 34- kDa mature PME gene lacking the PS region did not affect the vector virus expression (Fig. 1A3, bottom). Similarly, Fig. 1B shows that GFP production by crTMV:GFP vector was not affected by co-expression with proPME(fs) frameshift mutant. It is evident that various effects described above could not be due to the artifacts like *trans*-silencing between co-injected cDNA constructs that shared homology in their regulatory regions (e.g. the 35S promoter and Nos terminator).

The effect of proPME was studied in TMV and PVX VIGS systems, although in the case of PVX the effect was observed only if PVX was deficient in the 25 kDa gene-suppressor of RNAi (data not shown).

To elucidate, if the virus expression will be affected by various CW enzymes involved in pectin metabolism, the gene of *Nicotiana plumbaginifolia* glucuronyltransferase 1 (NpGUT1) [25] was used in co-agroinjection experiments. Expression of NpGUT1 had no effect on TMV:GFP reproduction in *N. benthamiana* leaves (Fig. 1C). It was found also that the inhibiting effect of proPME could be abolished by overexpression of the TMV MP gene. Coexpression of crTMV:GFP with proPME and TMV MP genes restored the crTMV:GFP reproduction (Fig. 1C). However, the real contribution of the MP-PME interactions to TMV infection is not clear yet.

3.2. Inhibition of proPME gene expression stimulates the TMV vector reproduction

We recently showed [18] that the CW-associated PME enzymatic activity was increased in *N. benthamiana* leaves by *Agrobacterium*-mediated expression of full-length proPME gene, whereas, PME activity was drastically suppressed in leaves agroinjected with antisense (asproPME) construct or with the mutant proPME (395A396A) gene encoding inactive enzyme. Furthermore, the TM domain of PME PS was found to be essential for PME delivery in the CW [18]. Here, we report that suppression of the cellular proPME expression stimulates the vectorvirus reproduction and GFP production. Firstly, inhibition of proPME expression by as-proPME significantly increased production of GFP by crTMV:GFP virus (Fig. 1A4). Secondly, the similar effect was observed when crTMV:GFP was co-expressed with the mutant proPME (395A396A) gene producing enzymatically inactive PME (Fig. 1C3). Thirdly, coagroinjection of crTMV:GFP with the deletion mutant A2-proPME (incapable of targeting the PME to CW) stimulated GFP expression by the vector virus (Fig. 1C4). Collectively, our results indicate that: (i) efficiency of TMV:GFP virus reproduction and GFP production depend inversely on

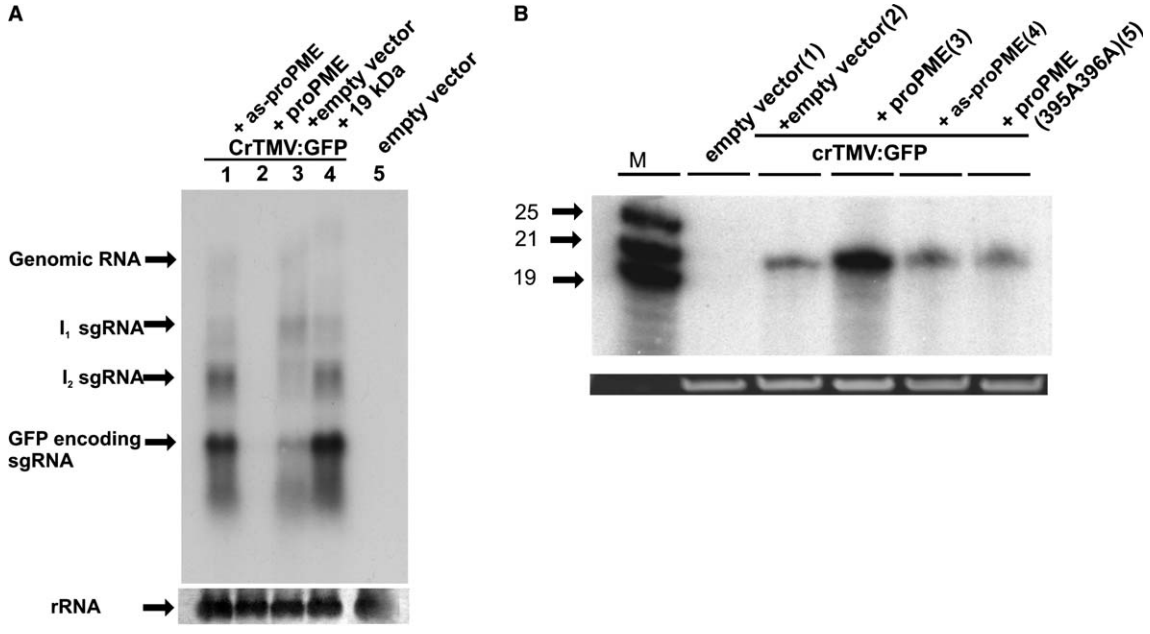

Fig. 2. ProPME-mediated suppression of the TMV-specific RNA production is accompanied by siRNA accumulation. (A) Northern blotting of crTMV:GFP RNAs isolated from leaves agroinjected with mixtures of constructs encoding: crTMV:GFP + as-proPME (1); crTMV:GFP + proPME (2); crTMV:GFP alone (3); crTMV + tombusvirus P19 gene (4); empty binary vector (control) (5). Positions of genomic and subgenomic RNAs are shown in the left. Equal loading in each lane is indicated by Methylene Blue staining of rRNA bands on membranes used for hybridization. (B) Analysis of crTMV:GFP-specific siRNAs after coagroinjection of crTMV:GFP and proPME and its derivatives (Northern Blotting with the GFP gene-sense probe). siRNA from leaves agroinjected with empty binary vector (1) or mixtures of binary vector containing: crTMV:GFP + empty vector (2), crTMV:GFP + proPME (3), crTMV:GFP + as-proPME (4); crTMV:GFP + proPME (396A397A) (5). Ethidium bromide staining of major RNA species in the short RNA samples is shown as a loading control.

the level of proPME gene expression; and (ii) enzymatically active CW-associated PME is important for inhibition of crTMV:GFP virus expression.

3.3. Degradation of virus-specific RNA and stimulation of siRNAs production by proPME gene co-expression

Fig. 2A (lane 2) shows that production of viral RNAs was drastically inhibited on proPME and crTMV:GFP co-expression. By contrast, inhibition of proPME synthesis by asproPME stimulated accumulation of GFP-specific subgenomic RNA (sgRNA) (Fig. 2A, lane 1). Similar effect was observed when crTMV:GFP genome was co-expressed with the gene P19 of TBSV tombusvirus, a well known suppressor of RNAi (Fig. 2A, lane 4). Rather low amount of genomic and intermediate size (I1 and I2) sgRNAs detected by Northern blotting was apparently due to degradation of the CP-unprotected viral RNA in virus-infected cells. In accordance with this explanation, a considerable amount of genomic RNA was revealed in leaves agroinjected with the CP-producing TMV-based vector (data not shown).

The ability to induce viral RNAs degradation suggested that proPME is involved by some way in the vector virus-induced RNA silencing. To gain further insight into this process, accumulation of virus-specific siRNAs was examined in joint agroinojections of crTMV:GFP with: (i) proPME; (ii) as-proPME; and (iii) enzymatically inactive proPME (395A396A) mutant. Fig. 2B (lane 3) shows that co-expression of proPME resulted in a significant increase in accumulation of the vector virus-specific siRNAs complementary to GFP gene. Thus, the transient expression of proPME in plant cells infected with a tobamovirus stimulated antiviral response manifested in the reduction of TMV-specific RNAs and increase of siRNA production as a hallmark of PTGS development. Collectively, these data provided evidence that, in additional to other functions, the multifunctional proPME plays a role of the enhancer of RNAi induced by crTMV:GFP.

3.4. PME stimulates RNA silencing of individual foreign gene

Next, we co-agroinjected *N. benthamiana* leaves by agrobacteria with binary vectors containing proPME and GFP genes. Northern blot hybridization with GFP-specific probe showed that expression of proPME decreased production of GFP mRNA (Fig. 3A2). Conversely, efficiency of siRNA production was markedly stimulated upon GFP + proPME expression (Fig. 3B2). In line with these observations, the amount of GFP protein accumulation was dropped down by several times (Fig. 3C2), whereas co-expression of GFP gene with as-proPME and proPME (395A396A) constructs increased accumulation of the GFP mRNA (Figs. 3A3 and A4) and the protein (Fig. 3C3 and C4). Remarkably, only negligible amounts of siRNA could be detected in these samples (Figs. 3B3 and B4). These data indicated that PME stimulates not only VIGS, but the RNAi of individual transgenes as well.

3.5. PME-induced relocation of DCL1 from nucleus to the cytoplasm

Certain of the nuclear cellular factors [26] are involved in RNAi and miRNA biogenesis (DCL1) [9,10], production of viral siRNA (DCL2) [10] and endogenous siRNA (DCL3) [10,11]. We examined the influence of proPME gene expression on intracellular localization of DCL1 and DCL2 (detected as a

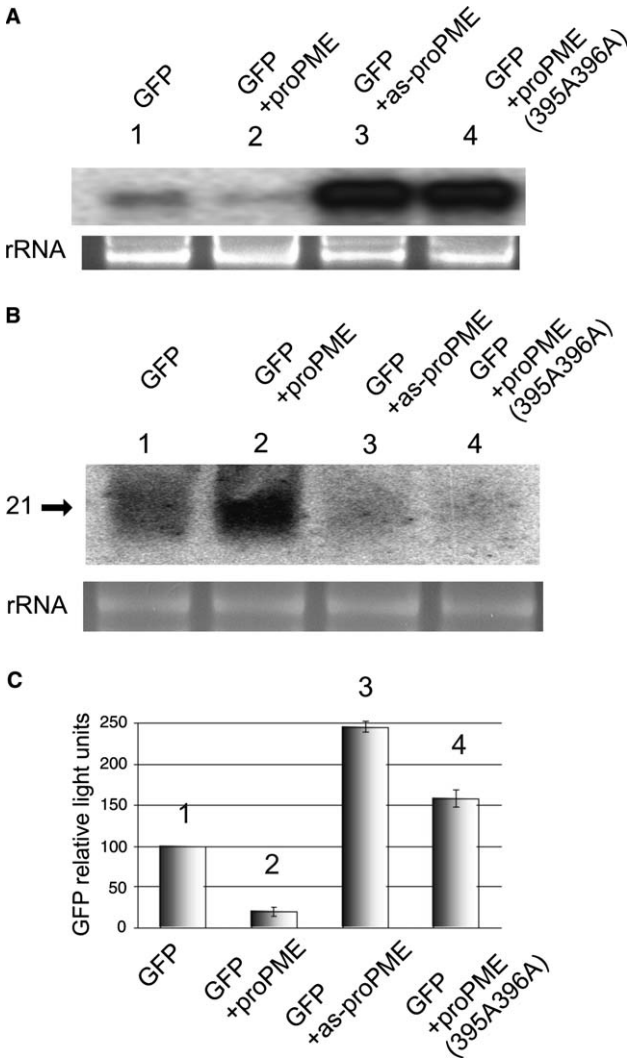

Fig. 3. PME enhances silencing of the transgene mRNA. (A) Northern analysis of RNA from *N. benthamiana* leaves co-agroinjected with GFP gene alone (1) or in the mixture with proPME gene (2) and its derivatives (3 and 4). After three days the total leaf RNA was isolated and tested by northern-hybridization with GFP-specific probe. rRNA bands (bottom) show loading control. (B) siRNAs accumulation after co-agroinjection of GFP and proPME genes. Arrow indicates position of the 21-nt oligoribonucleotide. (C) Fluorimetric analysis of GFP expression in leaves agroinjected with GFP gene alone (1) or in the mixture with proPME (2), as-proPME (3) or proPME (395A396A) (4).

DCL-GFP fusion). Figs. 4A and B show that expression in *N. benthamiana* leaves of DCL1:GFP alone resulted in exclusive fluorescence of nuclei. The same localization was observed in cells expressing GFP fused with the nuclear localization signal (data not shown). It is particularly noteworthy that co-expression of proPME changed the DCL1 intracellular distribution pattern drastically: only fluorescence of cytoplasm could be revealed, whereas nucleus staining was not detected (Figs. 4E and F). Different proPME gene modifications including as-proPME, proPME (395A396A) and A2-proPME had no influence on nuclear localization of DCL1:GFP (not shown). It could be proposed that proPME-triggered relocation of DCL1 to the cytoplasm is involved in the PMEmediated enhancement of RNAi. Expression of the DCL2:GFP fusion protein was detected both in nucleus and the cytoplasm (Figs.

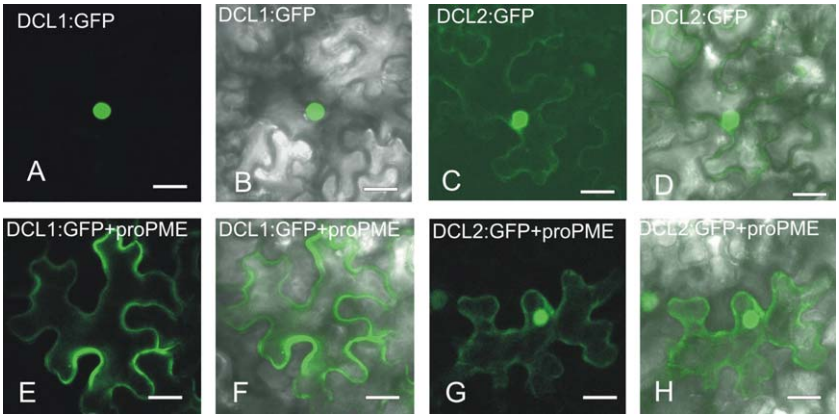

Fig. 4. PME induced relocation of nuclear DCL1 to the cytoplasm. Laser confocal scanning microscopy (A, C, E, G) and overlay of respective image and a false-transmission image (B, D, F, H) showing fluorescence in the cell wall limits of epidermal cells of *N. benthamiana* leaves agroinjected with DCL1:GFP or DCL2:GFP gene alone or in the presence of proPME gene. Scale bar = 20 μM.

4C and D) and its subcellular localization was not changed after co-injection with proPME (Figs. 4G and H).

3.6. ProPME activates miRNA biogenesis

To elucidate, whether the PME-mediated relocation of nuclear DCL1 to the cytoplasm could affect miRNA biogenesis, *N. benthamiana* leaves were co-agroinjected with binary vectors containing the proPME gene and the sequence of miR171 precursor (pBICmiR171prec) from *A. thaliana* [21]. Fig. 5A shows that co-expression of pBICmiR171precursor with a native proPME stimulated the 21-nt miRNA production, whereas, no stimulation of miRNA accumulation was observed under co-expression with enzymatically inactive proPME (395A396A). Furthermore, the results presented in Fig. 5B provided evidence that the pBICmiR171prec-mediated miRNA processing stimulated somewhat the vector virus expression (estimated as a sgRNA accumulation). The crTMV:GFP expression was restored to the level of control (vector virus taken alone) upon co-agroinjection pBICmiR171prec with proPME (Fig. 5B).

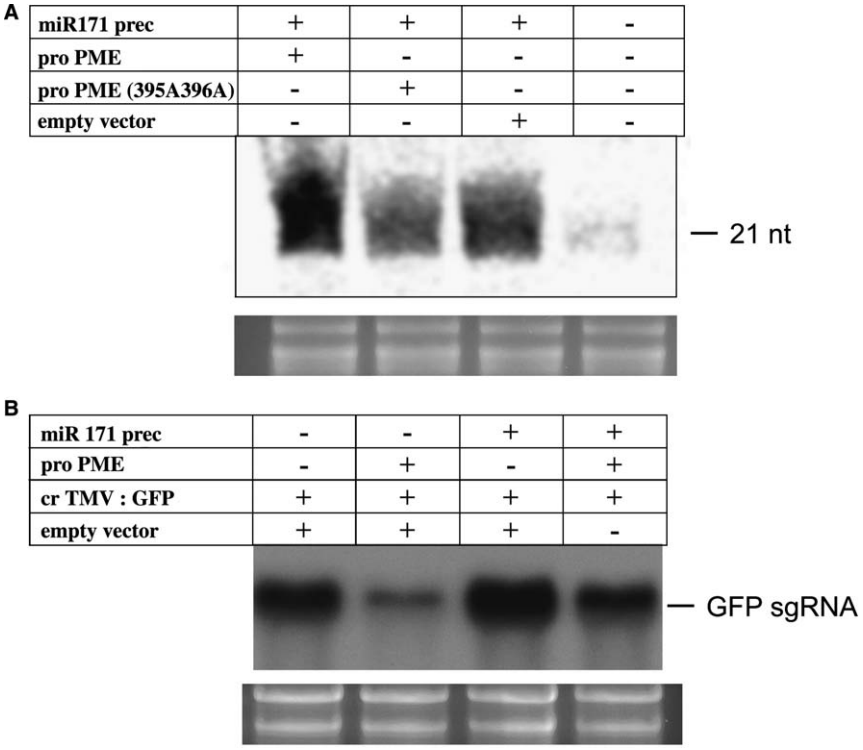

Fig. 5. The interplay between proPME gene expression, miRNA biogenesis and crTMV:GFP reproduction. (A) Activation of miRNA biogenesis by proPME. *N. benthamiana* leaves were coagroinjected with mixtures indicated above the panels. Short RNAs were analyzed at 2 dpi by Northern blotting with miR171-specific probe. *Agrobacterium* with empty vector was added to the mixture for maintaining equal concentration of specific vector. (B) Competition between miRNA processing and crTMV:GFP reproduction. Panel shows crTMV:GFP sgRNA isolated from leaves three days after agroinjection. The bottom shows rRNA bands as a loading control.

4. Discussion

There are at least three RNA silencing pathways for specific genes in plants: VIGS, TIGS and the silencing of endogenous messenger RNAs by miRNAs [1–5]. Agroinjection of the vector virus represents an example of the first pathway where cytoplasmic RNA silencing is accompanied by siRNA production [20]. In our study, the proPME-induced reduction in RNA and GFP production by the vector virus were invariably accompanied by a significant growth of siRNAs accumulation. The results indicated that efficiency of the vector virus reproduction and GFP production depended inversely on the level of proPME gene expression.

Reproduction of the virus vector was not affected by expression of the mature PME (Fig. 1A3) and even stimulated by A2-proPME mutant (Fig. 1C4) that lacked the CW-targeting ability. Apparently, the CW-associated enzymatic activity, rather than the amount of the PME protein produced, was important for the PME anti-viral activity (Figs. 1A and C). Overall, these results suggested that proPME-mediated inhibition of crTMV:GFP reproduction and GFP production was due to unexpected ability of proPME to enhance RNAi and the degradation of viral RNAs.

The proPME-mediated enhancement of RNA silencing is a novel function of this protein that can be universally applied to silencing RNAs of different viruses (TMV, PVX) as well as to mRNAs of individual foreign genes (Fig. 3). Thus, the PME gene expression resulted in degradation of monocistronic GFP mRNA, accumulation of GFP-specific siRNAs and inhibition of the GFP production (Figs. 3A–C).

The mechanism of proPME-mediated enhancement of RNAi is obscure. Our experiments showed that efficient subcellular redistribution of DCL1 consisting in relocation of DCL1 from nucleus to the cytoplasm was triggered by proPME gene expression (Figs. 4E and F). The functional role of this phenomenon in RNAi enhancement is not clear. It is tempting to assume that cytoplasmically localized DCL1 can be recruited for siRNA production and, therefore, for silencing enhancement. Our results also provided direct evidence that the proPME activated miRNA biogenesis (Fig. 5A). It could be hypothesized that DCL1 relocated to the cytoplasm may use as substrates both miRNA precursor and viral RNA. The pBICmiR171prec-mediated miRNA processing stimulated somewhat the vector virus expression; however this stimulation was abolished upon co-agroinjection of pBICmiR171prec with proPME. Recently, the evidence was provided [21] that, DCL localized in the nucleus participates in a plant virus RNA reproduction and is involved in biogenesis of both siRNA and miRNA. We speculate that the effect mentioned above was due to a competition for DCL1 between miRNA processing and crTMV:GFP reproduction.

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