PECTIN METHYLESTERASE ENHANCES *TOMATO BUSHY STUNT VIRUS* P19 RNA SILENCING SUPPRESSOR EFFECTS

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Abstract

A ubiquitous cell wall enzyme, pectin methylesterase (PME), a multifunctional protein which catalyzes pectin deesterification, producing atmospheric methanol, participates in cell wall modulation during general plant growth and pollen tube growth. The tobacco PME interacts with the movement protein of *Tobacco mosaic virus* (TMV) suggesting that PME may be involved in cell-to-cell movement of plant viruses, whereas methanol produced by PME-catalyzed reactions may act as a signal molecule in plant-plant and plant-pathogen interactions. Furthermore, PME also is an efficient enhancer of virus- and transgene-induced gene silencing (VIGS and TIGS, respectively) via activation of siRNA and miRNA production. Enhancement of VIGS and TIGS is accompanied by relocation of the DCL1 protein from the cell nucleus to the cytoplasm. This chapter shows that PME can prevent nuclear transport of reporter proteins, GFP and GFP-tagged TMV movement protein (MP) and MS2 phage coat protein fused with a nuclear localization signal (NLS). Furthermore, PME reduced nuclear transport of a plant protein ALY. Interestingly, this PME activity enhanced the effect of a plant virus RNA silencing suppressor, the *Tomato bushy stunt virus* (TBSV) P19 protein, which is known to relocate the host ALY to the cell

cytoplasm. PME production is elevated under stress conditions, and the invading virus may exploit the ability of PME to impair ALY nuclear transport for augmenting the activity of its P19 suppressor. Indeed, coexpression of PME and P19 substantially enhanced TMV infection. The crucifer-infecting TMV (crTMV) RNA accumulation was increased 10 times at 3 dpi in leaf areas co-agroinjected with P19 and PME. The GFP production in leaves infiltrated with crTMV:GFP together with binary vectors expressing PME and P19 was doubled in comparison with P19 alone. Because only enzymatically active PME, which is secreted into cell wall, was able to suppress nuclear import, we hypothesize that the products of pectin deesterification, demethylesterified homogalacturonan or methanol, may immediate the effects of PME on nuclear import.

Introduction

All animals and plants utilize small RNA molecules to control protein expression during different developmental stages and in response to viral infection. The multiple plant proteins involved in RNAi may be subdivided into the main and auxiliary proteins. The main proteins involved in the plant siRNA pathways, including RNA-dependent RNA polymerases (RDRs), dsRNA-binding proteins (DRBs), DCLs and AGOs, are members of large protein families [1-3]. Auxiliary proteins such as a JmjC domain-containing protein [4], GW repeat proteins [5], silencing defective 5 (SDE5) [6], SNF2 domain-containing proteins [7], Aly or Hin19 protein [8-11] modify VIGS and TIGS development. Identification of novel additional host proteins associated with RNAi would be helpful for understanding their contribution to gene silencing as a part of host defense mechanism.

A ubiquitous cell wall enzyme, PME, a secreted protein which catalyzes pectin deesterification, is shown to be involved in binding to the movement protein (MP) of TMV [12,13], suggesting that PME is engaged in the cell-to-cell movement of plant viruses [14]. A novel function of PME as an efficient enhancer of RNA silencing was shown recently [15]. Co-agroinjection of *Nicotiana benthamiana* leaves with the proPME gene and the TMV:GFP vector

resulted in a stimulation of VIGS manifested by inhibition of GFP production, viral RNA degradation, and stimulation of siRNAs production. The 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. Moreover, the suppression of TMV short- and long-distance movement was observed in PME transgenic plants [16]. It was hypothesized that relocated to the cytoplasm DCL1 may use both miRNA precursor and viral RNA as substrates.

This chapter shows that PME as an efficient enhancer of VIGS and TIGS can prevent nuclear traffic of the plant protein ALY which relocates the TBSV silencing suppressor P19 protein into the cell nucleus. PME-induced inhibition of ALY:GFP nuclear transport and enhanced P19 silencing suppression effects, resulting in substantial increase in TMV reproduction.

PME blocks nuclear transport of NLS-containing proteins

To understand whether PME-mediated DCL1 relocation is a specific event or PME interferes with nuclear proteins in general, we first examined intracellular localization of an NLS-containing reporter, GFP:NLS, expressed in the absence or presence of PME. Fig. 1A shows that free GFP expressed in *N. benthamiana* leaves partitions between of the nucleus and the cytoplasm, due to its small size, which is below diffusion limit of the nuclear pore. Also as expected, GFP fused to the prothymosin α NLS accumulated exclusively in the cell nucleus (Fig.1B).

Nuclear localization of GFP:NLS was largely impaired by coexpression of a full-length PME, resulting in appearance of a significant pool of cytoplasmic GFP:NLS (Fig. 1C). Epifluorescent microscopy analysis showed that coincubation of the GFP:NLS-expressing leaf with a PME-expressing leaf resulted in significant accumulation of percentage of cells with GFP fluorescence in cytoplasm as compared to coincubation with an vector control (93.8±0.60 vs. 15.5±1.34). This effect of PME expression was not specific only for GFP:NLS. We tested two

other unrelated proteins tagged with GFP and fused to SV40 NLS: MS2 bacteriophage CP (GFP:NLS:CP_{MS2}) [17] and TMV MP (MP:GFP:NLS). Fig. 1D shows that GFP:NLS:CP_{MS2}, indeed, accumulated in the cell nucleus, and PME coexpression impeded this accumulation (Fig. 1E) in more than 90% cells (vs. 10% in control). As GFP:NLS:CP_{MS2} is larger than free GFP, it did not diffuse into the nucleus (compare panels C and E in Fig. 1) and remained exclusively cytoplasmic. When wild-type TMV MP tagged with GFP was expressed in leaf tissue, we observed clearly distinguishable irregular cortical bodies, and cell wall-associated puncta, characteristic of the well-known ER and plasmodesmata-specific localization of this protein (Fig. 1F). Fusion with NLS directed virtually all MP:GFP into the cell nucleus (Fig. 1G), indicating that this signal overcomes the putative plasmodesmata-targeting activity of MP. Importantly, coexpression of PME partially restored the characteristic wild-type pattern of MP localization (Fig. 1H), suggesting that the nuclear import of MP:GFP:NLS was significantly impaired. In control experiments, coexpression of another cell wall protein NtGUT1 [18] did not alter nuclear accumulation of all three NLS-containing reporters (data not shown). Furthermore, PME mutants, PME (396A397A) and A2-PME, which lack the enzymatic activity [19], also showed no effect on nuclear import of the reporters (data not shown). We then used an MP mutant, Del4, which lacks the plasmodesmata-dilating domain [20], to demonstrate that inhibition of MP:GFP:NLS nuclear import by PME is not dependent on MP interaction with plasmodesmata. Fig. 1 shows that Del4 tagged with GFP exhibited predominantly cytoplasmic and ER-associated localization (panel I) and relocalized to the cell nucleus upon fusion to SV40 NLS (panel J). Coexpression of PME largely blocked nuclear import of Del4:GFP:NLS (Fig. 1K). Similarly to MP:GFP:NLS, nuclear import of Del4:GFP:NLS was not affected by inactive mutants of PME or by NtGUT1 (data not shown). Next, we used western blot analyses to address the possibility that the reporter protein may have undergone proteolytic cleavage which may result in cytoplasmic accumulation of the cleavage products. These experiments (data not shown)

detected no cleavage products of intracellular MP:GFP:NLS or Del4:GFP:NLS following expression alone or coexpression with PME, its mutant derivatives, or NtGUT1.

PME relocates the plant nuclear protein ALY and enhances the RNA silencing suppressor activity of TBSV P19

As PME interfered with the nuclear import of reporters containing a model NLS, most likely it also can affect import of at least some cellular nuclear proteins. Indeed, PME expression impaired nuclear import of the plant mRNA nuclear export factor ALY [8]. Nuclear import of ALY, in turn, is known to correlate with the biological activity of the TBSV silencing suppressor, P19, with which it interacts; if ALY succeeds to direct associated P19 into the cell nucleus, then P19 is rendered inactive, but if P19 sequesters ALY in the cell cytoplasm, it remains capable to suppress silencing [8,10]. Consistent with this model, PME-induced inhibition of ALY nuclear import enhanced silencing suppression by P19, resulting in substantial increase in viral reproduction. Fig. 2A shows that GFP-tagged ALY expressed in plant leaves accumulated in the cell nucleus. Coexpression of PME altered this pattern of ALY:GFP subcellular localization, redirecting most of it to the cell cytoplasm (Fig. 2B) with significant accumulation of percentage of cells with GFP fluorescence in cytoplasm as compared to coexpression with vector control (87.3±0.51 vs. 11.5±1.43). Within the time course of the experiment, negative controls, an anti-sense PME (asPME) construct and the construct expressing enzymatically inactive PME (396A397A), had no effect on the ALY:GFP nuclear accumulation (Fig. 2C, D, respectively). This change in ALY subcellular localization promoted by PME expression resembled that induced by coexpression of TBSV P19 [8] (Fig. 2E). PME or P19 relocated virtually all detectible ALY:GFP, thus coexpression of both PME and P19 with ALY:GFP had no further discernable effect on ALY:GFP localization (Fig. 2F).

As PME effect on ALY subcellular localization mimics that of P19 and P19 function in plant cells may correlate with its ability to interact with ALY and change its intracellular localization [8], we examined whether exogenous PME may also enhance silencing suppressor

activity of P19. To this end, we agroinfiltrated leaves with a GFP-tagged, movement-deficient mutant of crucifer-infecting TMV (crTMV), crTMV:GFP-MP(fs); coinfiltration of a tested protein with crTMV:GFP-MP(fs) allows to estimate the effects on silencing by counting the number of cells displaying GFP fluorescence. Confirming our previous observations that PME is able to promote gene silencing [15], coinfiltration of crTMV:GFP-MP(fs) and PME reduced the number of transformed cells exhibiting the GFP signal (Fig. 3A) twofold, as compared to crTMV:GFP-MP(fs) expressed alone (Fig. 3B). Compared to the same control, coinfiltration with P19 increased the number of the GFP-expressing cells also twofold, indicating silencing suppression (Fig. 3B). Joint expression of crTMV:GFP-MP(fs) with both P19 and exogenous PME increased the level of GFP accumulation by a factor of four (Fig. 3B), suggesting that PME enhances the silencing suppressor effect of P19.

Fig. 3C shows that joint injection of P19 and PME drastically increased accumulation of another marker RNA, crTMV:GFP with wild-type MP. Our analysis of the GFP amounts in the agroinfiltrated tissues confirmed that PME indeed suppressed GFP expression as compared to crTMV:GFP agroinfiltrated alone, but it substantially increased this expression in the presence of both P19 and PME. As expected this enhanced GFP expression was not observed when P19 was coinfiltrated with the asPME construct (Fig. 3D).

Furthermore, PME had no such effects on an unrelated viral silencing suppressor HC-Pro, the activity of which does not involve nuclear import (not shown). It is tempting to speculate that TBSV has evolved to take the advantage of plant stress and defense response which includes induction of PME expression. In this scenario, the host plant responds to the viral invasion via mechanical inoculation, for example,, by inducing PME synthesis whereas the virus uses the resulting decrease in nuclear import to circumvent ALY nuclear uptake to inactivate the viral P19 suppressor.

The mechanism by which PME affects nuclear import is unknown. PME itself is a cell wall-associated protein, and only enzymatically-active PME capable of targeting to the cell wall

[15,19] could impair nuclear import. Thus, it is likely that PME does not interfere with the nuclear import machinery directly; instead, the products of PME-mediated pectin deesterification, such as methanol [21] and other metabolites, may serve mediate the PME effect on nuclear import.

Acknowledgements

We thank members of the MSU Department of Virology for helpful discussions and technical assistance. This work was partly supported by grants from RFBR, Moscow Government and Icon Genetics GmbH. The work in the VC laboratory is supported by grants from NIH, NSF, NIFA, BARD, and BSF.

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Figure legends

Figure 1. PME inhibits nuclear import of NLS-containing proteins: GFP, GFP:CP_{MS2} and TMV MP:GFP.

(A) GFP. (B) GFP:NLS. (C) GFP:NLS coexpressed with PME. (D) GFP:NLS:CP_{MS2}. (E) GFP:NLS:CP_{MS2} coexpressed with PME. (F) MP:GFP. (G) MP:GFP:NLS. (H) MP:GFP:NLS coexpressed with PME. (I) Del4:GFP. (J) Del4:GFP:NLS. (K) Del4:GFP:NLS coexpressed with PME. Images are projections of several confocal sections. Panel D represents a confocal image superimposed on a bright field image of the same cell. Bars = 20 μm.

Figure 2. PME relocates ALY from the cell nucleus to the cytoplasm.

(A) ALY:GFP. (B) ALY:GFP coexpressed with PME. (C) ALY:GFP coexpressed with asPME. (D) ALY:GFP coexpressed with PME(396A397A). (E) ALY:GFP coexpressed with P19. (F) ALY:GFP coexpressed with P19 and PME. Images are projections of several confocal sections superimposed on a bright field image of the same cell. Bars = 20 μm.

Figure 3. PME enhances the RNA silencing suppressor activity of TBSV P19.

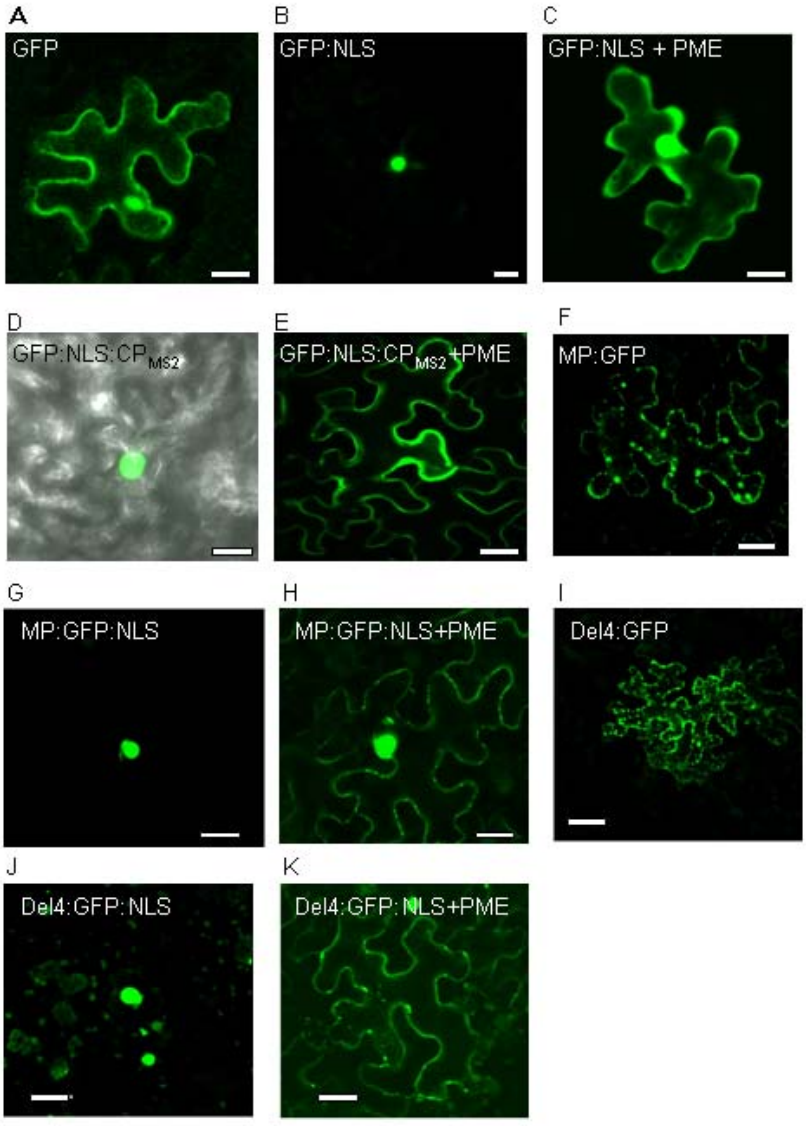
(A) Agroinfiltration experiments with a GFP-tagged, movement-deficient mutant crTMV:GFP-MP(fs). Visualization of GFP expression in leaf areas coinfiltrated with crTMV:GFP-MP(fs) and an empty, control binary vector (V) or binary vectors expressing PME, P19, or both PME and P19. GFP signal is in green and plastid autofluorescence is in magenta. Bars = 200 μm.

(B) Numbers of GFP-expressing cells within the leaf areas shown in (A). The data represent mean values with standard error for 5-8 independent experiments.

(C) Relative quantity of crTMV RNA at 3 dpi as determined by quantitative real-time PCR in leaf areas agroinjected either by the GFP-expressing vector alone (control) or together with P19 or P19 with PME (co-injection).

(D) GFP expression at 10 dpi in leaves infiltrated with crTMV:GFP alone (control) or together with binary vectors expressing the indicated the expressing PME, P19, both PME and P19, or asPME. Protein was detected by polyacrylamide gel electrophoresis and Coomassie blue staining. Equal loading of all protein samples is indicated by the intensity of the Rubisco protein band. Numbers above gel wells are relative GFP band density units.

Figure 1.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C
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D
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G
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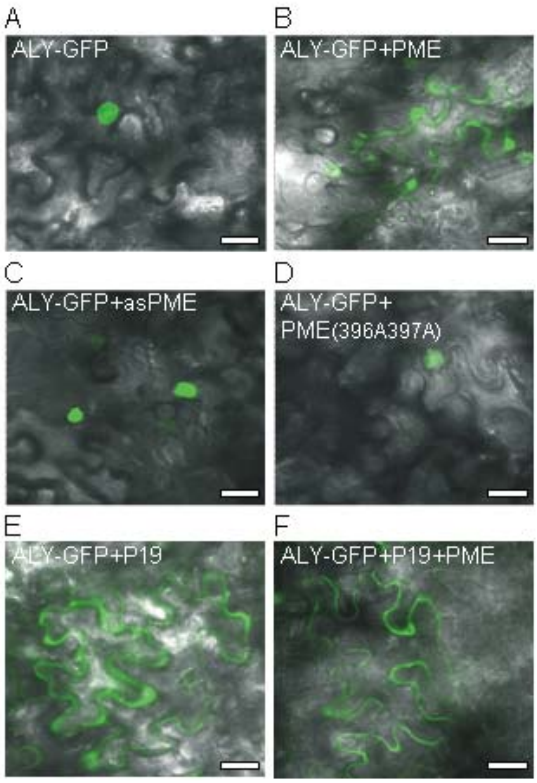
Figure 2.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B
ALY-GFP+PME
C
ALY-GFP+asPME
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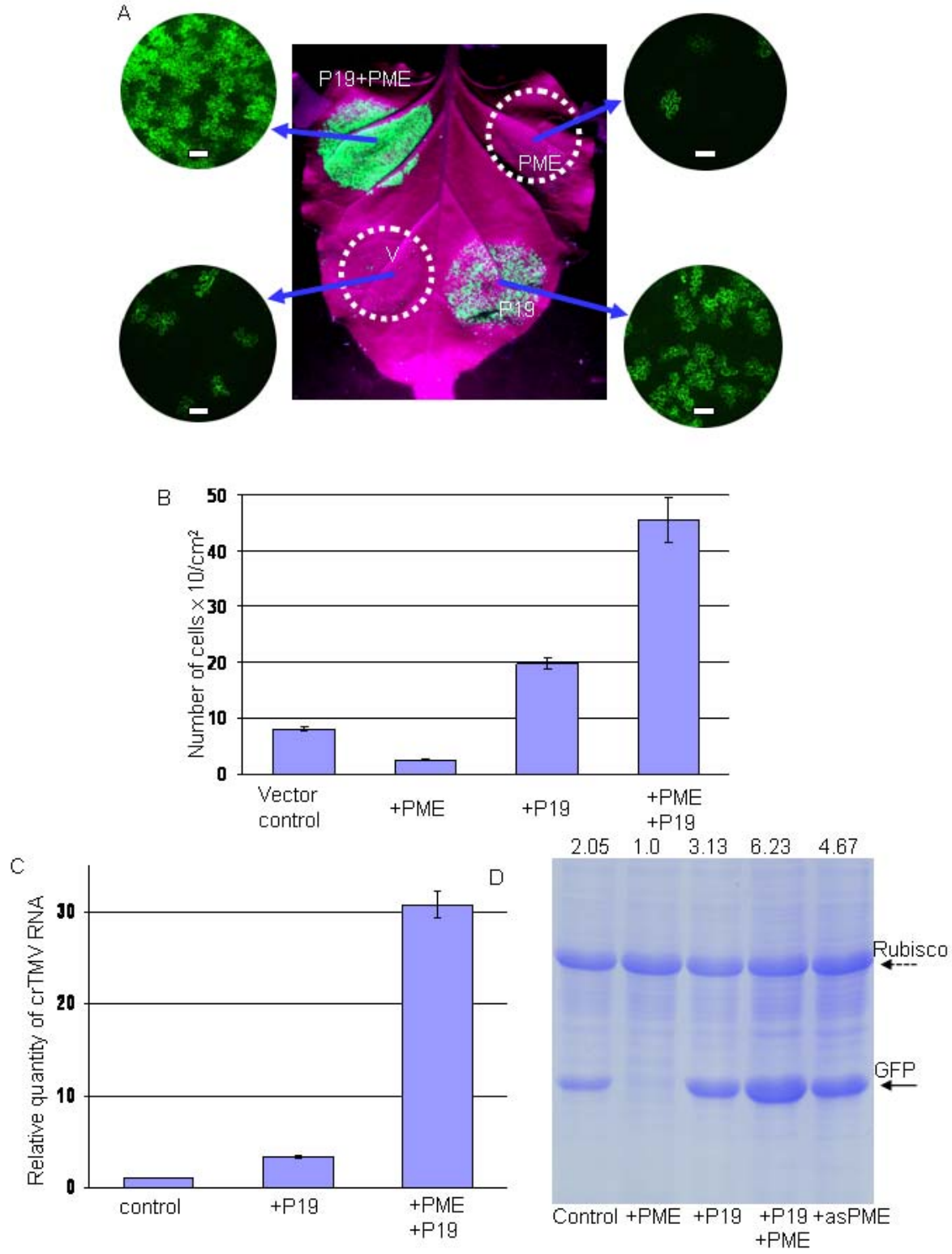
F
ALY-GFP+P19+PME

Figure 3.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PME
V
P19
B
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10
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Vector control
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+P19
+PME +P19
C
Relative quantity of crTMV RNA
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10
0
control
+P19
+PME +P19
D
2.05
1.0
3.13
6.23
4.67
Rubisco
GFP
Control
+PME
+P19
+P19 +PME
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