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1 **rgs-CaM Detects and Counteracts Viral RNA Silencing Suppressors in Plant**

2 **Immune Priming**

3

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19

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21

22 **ABSTRACT**

23 Primary infection of a plant with a pathogen that causes high accumulation of salicylic  
24 acid in the plant typically via a hypersensitive response confers enhanced resistance  
25 against secondary infection with a broad spectrum of pathogens, including viruses. This  
26 phenomenon is called systemic acquired resistance (SAR), which is a plant-priming for  
27 adaption to repeated biotic stress. However, the molecular mechanisms of SAR-  
28 mediated enhanced inhibition, especially of virus infection, remain unclear. Here, we  
29 show that SAR against cucumber mosaic virus (CMV) in tobacco plants (*Nicotiana*  
30 *tabacum*) involves a calmodulin-like protein, rgs-CaM. We previously reported the  
31 antiviral function of rgs-CaM, which binds to and directs degradation of viral RNA  
32 silencing suppressors (RSSs), including CMV 2b, via autophagy. We found that rgs-  
33 CaM-mediated immunity is ineffective against CMV infection in normally growing  
34 tobacco plants but is activated as a result of SAR induction via salicylic acid signaling.  
35 We then analyzed the effect of overexpression of rgs-CaM on salicylic acid signaling.  
36 Overexpressed and ectopically expressed rgs-CaM induced defense reactions including  
37 cell death, generation of reactive oxygen species, and salicylic acid signaling. Further  
38 analysis using a combination of salicylic acid analogue BTH and Ca<sup>2+</sup> ionophore,  
39 A23187, revealed that rgs-CaM functions as an immune receptor that induces salicylic  
40 acid signaling by simultaneously perceives both viral RSS and Ca<sup>2+</sup> influx as infection  
41 cues, implying its autoactivation. Thus, secondary infection of SAR-induced tobacco  
42 plants with CMV seems to be effectively inhibited through 2b recognition and  
43 degradation by rgs-CaM, leading to reinforcement of antiviral RNA silencing and other  
44 salicylic acid-mediated antiviral responses.

45

46 **IMPORTANCE**

47 Even without an acquired immune system like that in vertebrates, plants show enhanced  
48 whole-plant resistance against secondary infection with pathogens; this so-called  
49 systemic acquired resistance (SAR) has been known for more than half a century and  
50 continues to be extensively studied. SAR-induced plants strongly and rapidly express a  
51 number of antibiotics and pathogenesis-related proteins targeted against secondary  
52 infection, which can account for enhanced resistance against bacterial and fungal  
53 pathogens but are not thought to control viral infection. This study showed that  
54 enhanced resistance against cucumber mosaic virus is caused by a tobacco calmodulin-  
55 like protein, rgs-CaM, which detects and counteracts the major viral virulence factor  
56 (RNA silencing suppressor) after SAR induction. rgs-CaM-mediated SAR illustrates  
57 the growth vs. defense trade-off in plants, as it targets the major virulence factor only  
58 under specific biotic stress conditions, thus avoiding the cost of constitutive activation  
59 while reducing the damage from virus infection.

60

61 **INTRODUCTION**

62 Being sessile, plants are exposed to pathogen attacks and diverse environmental stresses  
63 and are unable to evade exposure to subsequent attacks. Instead, plants retain the  
64 “memory” of experiences with pathogens and environmental stresses, enabling them to  
65 mount defense reactions to subsequent challenges more effectively. A number of  
66 antibiotics and pathogenesis-related proteins targeted against secondary infection are  
67 expressed more strongly and rapidly. This general phenomenon is called priming (1);  
68 priming induced by and against pathogens is called systemic acquired resistance (SAR)  
69 (2). SAR was discovered decades ago (3, 4) and has the potential to confer on crops

70 enhanced resistance against diverse pathogens; for this reason, induction of SAR using  
71 chemical and biological agents has been explored. Studies in recent decades have  
72 dramatically unveiled the molecular mechanisms of SAR (2). SAR-induced plants  
73 systemically accumulate salicylic acid (5), an important phytohormone for mediating  
74 immune responses to pathogens (6, 7), including viruses (8). In *Arabidopsis thaliana*,  
75 the primed state of SAR is partly attributed to the action of the genes encoding the non-  
76 expressor of pathogenesis-related proteins NPR1, NPR3, and NPR4, which have been  
77 shown to be salicylic acid receptors and mediators (9-12). In addition, epigenetic  
78 modifications in SAR-induced plants have been suggested to be involved in the primed  
79 state (13). The existence of transgenerational SAR (14) supports the involvement of  
80 epigenetic modifications because such modifications can be inherited in plants (15).  
81 Thus, the requirement of NPR1 for transgenerational SAR (14) implies that salicylic  
82 acid is also involved in the epigenetic modifications. Although systemic salicylic acid  
83 biosynthesis (i.e., including plant parts distant from the site of infection) is required for  
84 induction of SAR (6), salicylic acid derivatives and other chemical molecules recently  
85 have been identified as the systemic signaling molecules (5).

86         In contrast to our understanding of the mechanisms of how SAR is induced and  
87 maintained, even across generations, the exact mechanisms underlying the enhanced  
88 resistance against pathogens, especially viruses, at secondary infection sites in SAR-  
89 induced plants remain to be examined. One such mechanism may be RNA silencing, a  
90 major plant defense against diverse viruses, which is induced by double-stranded RNA  
91 (dsRNA) and targets its cognate RNAs for degradation (16, 17). RNA silencing and  
92 salicylic acid-mediated immunity cooperatively inhibit systemic infection by the plum  
93 pox virus (18). RNA-dependent RNA polymerase 1, which is involved in antiviral

94 immunity through its role in RNA silencing (19-23), is induced by salicylic acid (22,  
95 23). The RNA silencing components dsRNA binding protein 4, Argonaute 2 (AGO2),  
96 and AGO4 are involved in salicylic acid-mediated and nucleotide-binding site (NB)-  
97 leucine-rich repeat (LRR)-mediated immunity (24-26). On the other hand, resistance  
98 against cucumber mosaic virus (CMV) and tobacco mosaic virus was enhanced by  
99 applying exogenous salicylic acid to an *A. thaliana* triple mutant of the Dicer-like genes  
100 that was considered to completely lack antiviral RNA silencing, implying that SAR is  
101 independent of RNA silencing (27).

102         In this study, we revealed that a tobacco calmodulin-like molecule (a regulator  
103 of gene silencing calmodulin-like protein, thus designated rgs-CaM), is involved in  
104 SAR against CMV. rgs-CaM was initially isolated in a screen of tobacco proteins that  
105 interact with the helper component-proteinase (HC-Pro) of the tobacco etch virus (28).  
106 HC-Pro is a multifunctional protein found in viruses that are members of the genus  
107 *Potyvirus* and functions as an effector molecule that suppresses antiviral RNA silencing  
108 (RNA silencing suppressor [RSS]) (29-31). In a previous study, rgs-CaM was shown to  
109 be an endogenous RSS that suppresses virus-induced gene silencing (VIGS) by the  
110 potato virus X (PVX) vector, which was developed from a member of the genus  
111 *Potexvirus* (28). We and other groups confirmed that rgs-CaM has RSS activity (32-34)  
112 and facilitates infection by viruses in the genus *Begomovirus* via its RSS activity (34,  
113 35). However, we also observed an antiviral function of rgs-CaM: it binds to and directs  
114 degradation of two viral RSSs, HC-Pro and CMV 2b, via autophagy, resulting in  
115 reinforcement of antiviral RNA silencing in virus-infected cells (32). The present study  
116 reconciled these antagonistic functions of rgs-CaM by revealing a phase change in the  
117 rgs-CaM function: the antiviral function is dormant in normally growing plants and

118 activated after SAR is induced. Moreover, we found that rgs-CaM also functions as an  
119 immune receptor. Previously, necrotic symptoms and hypersensitive responses  
120 accompanied by programmed cell death were thought to be required for SAR induction  
121 (36). More recently, however, immune receptors, receptor-like kinases (RLK), and NB-  
122 LRR proteins, which mainly perceive pathogen invasion and mount defense responses  
123 in plants, have been shown to induce SAR via defense signaling regardless of whether  
124 cell death occurs (37, 38). In this study, we showed that rgs-CaM induces salicylic acid  
125 signaling via simultaneous perception of both viral RSS and calcium ion ( $\text{Ca}^{2+}$ ) influx  
126 as virus infection cues, implying autoactivation of the antiviral function of rgs-CaM in  
127 SAR. This study shows that two conditional reactions of tobacco plants (*Nicotiana*  
128 *tabacum*) against CMV — recognition of CMV infection, which induces salicylic acid  
129 signaling, and inhibition of CMV infection after SAR induction — are mediated by a  
130 single host protein.

131

## 132 **RESULTS**

### 133 **Overexpressed and ectopically expressed rgs-CaM induces cell death and defense** 134 **reactions**

135 We became aware of the association between rgs-CaM and other defense reactions other  
136 than RNA silencing, by observing transgenic tobacco plants that constitutively  
137 overexpressed the *rgs-CaM* gene under the control of the cauliflower mosaic virus  
138 (CaMV) 35S promoter. Among a dozen transgenic lines, two showed dwarfing,  
139 deformation, and partial necrosis on their leaves (Fig. 1Ai, B and C). These phenotypes  
140 were similar to those of lesion mimic mutants that involve hypersensitive response-like  
141 programmed cell death, which are accompanied by induction of reactive oxygen species

142 (ROS) and immune signaling components, including salicylic acid (39, 40). In the  
143 transgenic plants showing these phenotypes, cell death was observed (Fig. 1B), ROS  
144 were generated (Fig. 1C), and mRNA of the gene for pathogenesis-related protein 1a  
145 (*PR1a*), an indicator of activation of salicylic acid signaling (41), was induced in the  
146 leaves (Fig. 1Di, ii). The severity of the lesion mimic phenotype (Fig. 1Aii) and *PR1a*  
147 levels (Fig. 1Di, ii) varied both among and within rgs-CaM-overexpressing lines. These  
148 results with the previous our inoculation test that showed the enhanced resistance  
149 against CMV in the Line rgs-CaM16 (32) indicate the possibility that the overexpressed  
150 rgs-CaM can induce cell death and immune responses and signaling, though it does not  
151 always do so. We confirmed this possibility by two additional experiments.

152         First, rgs-CaM was overexpressed in wild-type tobacco plants by infection with  
153 a PVX vector expressing rgs-CaM. Infection with this vector caused necrotic spots,  
154 whereas infection with the empty PVX vector or the vector expressing the rgs-CaM  
155 gene that lacks the initiation codon to express its encoded protein [PVX-rgs-CaM(-atg)]  
156 did not (Fig. 2A). *PR1a* was induced significantly in leaves inoculated with the PVX  
157 vector expressing rgs-CaM but not in leaves inoculated with either the empty PVX  
158 vector or PVX-rgs-CaM(-atg). Second, rgs-CaM was transiently expressed in  
159 protoplasts prepared from wild-type tobacco leaves. Protoplasts transfection with an  
160 expression cassette containing *rgs-CaM* under the control of the CaMV 35S promoter  
161 resulted in cell death and ROS generation (Fig. 2B and C). In contrast, protoplasts  
162 transfected with negative control expression cassette [rgs-CaM(-atg)] did not  
163 significantly increase cell death or ROS generation. Taken together, these data suggest  
164 that overexpressed and ectopically expressed rgs-CaM induces immune responses and  
165 salicylic acid signaling.



166

167 **rgs-CaM is involved in salicylic acid signaling in response to CMV-Y infection**

168 Because overexpressed and ectopically expressed rgs-CaM induced immune responses  
169 and salicylic acid signaling in transgenic plants (Figs 1 and 2), we assume that  
170 endogenous rgs-CaM is also involved in induction of these responses, including  
171 salicylic acid signaling. Viral infection induces various immune responses and signals  
172 that are mediated via phytohormones, including salicylic acid, and thus rgs-CaM may  
173 be involved in these responses. We tested this possibility using PVX and CMV.

174         When rgs-CaM–knockdown tobacco plants, in which rgs-CaM was suppressed  
175 by an inverted repeat (IR) transgene (32), were inoculated with PVX, the levels of PVX  
176 coat protein (CP) and genomic and subgenomic RNAs (*gPVX* and *sgPVX*) observed by  
177 western and northern blotting, respectively. *sgPVX* was similar to those in inoculated  
178 wild-type tobacco plants but CP and *gPVX* accumulated to a lesser extent (Fig. 3A). We  
179 re-examined whether rgs-CaM facilitates or inhibits PVX infection using real-time PCR  
180 with more individual plants for each genotype ( $n = 8$ ). Two primer pairs to amplify  
181 cDNAs of PVX RNAs were used (Fig. 3B). One was designed to amplify the cDNA  
182 from PVX genomic RNA (RdRp) and another to amplify the cDNA from both genomic  
183 and subgenomic RNAs of PVX (CP). PVX RNAs accumulated slightly more in  
184 inoculated leaves of the rgs-CaM – knockdown plants, but a statistically significant  
185 difference was detected only for RdRp cDNA, indicative of PVX genomic RNA (Fig.  
186 3B). In non-inoculated upper leaves, PVX RNAs appeared to accumulate more in the  
187 rgs-CaM–knockdown plants than in wild-type plants, but the difference was not  
188 statistically significant. We then examined whether salicylic acid signaling was induced

189 in these plants by examining the mRNA level of *PR1a*. The *PR1a* mRNA level  
190 increased slightly but significantly in non-inoculated upper leaves of wild-type tobacco  
191 plants (Fig. 3C). Similar results were obtained in the *rgs-CaM*-knockdown plants but  
192 the differences with the wild-type plants were not significant. Our results suggest that,  
193 even if *rgs-CaM* is involved in defense and induction of salicylic acid signaling against  
194 PVX infection, its contribution is minimal. Reduced *rgs-CaM* mRNA levels were not  
195 observed in mock-inoculated leaves of the *rgs-CaM*-knockdown plants in comparison  
196 to those of wild-type plants though it reduced in *rgs-CaM*-knockdown plants in the  
197 other cases (Fig. 3D). In a previous study, we obtained several lines of *rgs-CaM*-  
198 knockdown plants (32) but could not propagate them because of their infertility. In the  
199 *rgs-CaM*-knockdown tobacco plants used in the present study, we speculate that *rgs*-  
200 *CaM* expression was not as severely suppressed and thus this line was fertile.

201 In contrast to the situation with PVX, we obtained quite different results with the  
202 CMV Y strain (CMV-Y). CMV RNAs and CP accumulated to similar levels in both  
203 wild-type and *rgs-CaM* knockdown tobacco plants (Fig. 4A). *PR1a* expression was  
204 strongly induced in CMV-inoculated leaves of wild-type tobacco plants, but to a lesser  
205 extent in the *rgs-CaM*-knockdown plants (Fig. 4B). Although there was no statistically  
206 significant difference in *PR1a* levels in inoculated leaves between wild-type and *rgs*-  
207 *CaM*-knockdown plants in the experiment shown in Fig. 4B, experiment 1 ( $n = 3$ ), we  
208 repeated the experiment with more samples ( $n = 9$ ) and detected a significantly higher  
209 *PR1a* level in the wild-type plants than in the knockdown plants (Fig. 4B, experiment  
210 2). Moreover, reduced *PR1a* expression in CMV-Y-inoculated leaves of the *rgs-CaM*-  
211 knockdown plants, compared with that in wild-type tobacco plants, was also detected  
212 previously (32). However, *PR1a* mRNA levels in the upper leaves of plants infected

213 with CMV-Y (Fig. 4B) and in leaves inoculated with CMV that lacked the 2b RSS  
214 (CMV $\Delta$ 2b) (Fig. 4D) were not lower in the rgs-CaM–knockdown plants than those in  
215 wild-type plants. This was even though CMV RNAs and CP accumulated similarly in  
216 both wild-type and rgs-CaM knockdown plants (Fig. 4C). Considering that rgs-CaM  
217 physically interacts with the dsRNA binding site of 2b (32) and is a calmodulin-like  
218 protein with EF-hand motifs that bind to Ca<sup>2+</sup> and probably transduce the Ca<sup>2+</sup> signal  
219 (42), these results led us to hypothesize that rgs-CaM is an immune receptor. According  
220 to our model, in CMV-Y-infected epidermal cells in an inoculated leaf, 2b is expressed  
221 by CMV-Y, Ca<sup>2+</sup> influx is derived from wounding caused by mechanical inoculation  
222 with Carborundum (Fig. 5A), and salicylic acid signaling is reduced by knocking down  
223 of rgs-CaM (Fig. 4B, experiment 2). However, a non-inoculated upper leaf (Fig. 5B)  
224 and a leaf inoculated with CMV $\Delta$ 2b (Fig. 5C) lack either 2b expression or Ca<sup>2+</sup> influx,  
225 and salicylic acid signaling (*PR1a* expression) is not reduced by knocking down of rgs-  
226 CaM (Fig. 4B and D). Therefore, we hypothesize that rgs-CaM induces salicylic acid  
227 signaling through perception of both 2b and Ca<sup>2+</sup> influx as cues of the initial infection  
228 with CMV-Y in inoculated leaves.

229

### 230 **rgs-CaM induces salicylic acid signaling via perception of both Ca<sup>2+</sup> and viral RSS**

231 To examine this hypothesis, we used transgenic tobacco plants that constitutively  
232 express a viral RSS, i.e., either CMV 2b or HC-Pro of clover yellow vein virus  
233 (CIYVV); the latter was chosen because HC-Pro is known to interact with rgs-CaM (28,  
234 32). We previously showed that the *PR1a* mRNA level did not increase in these  
235 transgenic tobacco plants, compared with that in wild-type tobacco plants though the  
236 *rgs-CaM* mRNA level somewhat increased in transgenic plants (32). *PR1a* expression

237 was monitored at different times in the transgenic tobacco plants after wounding stress  
238 caused by opening microperforations in leaves with a bundle of about 400 pins (Fig.  
239 5D). *PR1a* expression was induced at a level detectable by RT-PCR in the transgenic  
240 plants expressing 2b and HC-Pro 24 h after wounding, but not in wild-type plants (Fig.  
241 5E).

242 Wounding causes various changes and reactions associated with morphological  
243 damage in injured cells and surrounding cells, including  $\text{Ca}^{2+}$  influx and generation of  
244 ROS. In fact, ROS were generated at the wounding sites in leaves of both wild-type  
245 plants and transgenic tobacco plants expressing viral RSSs (Fig. 5D). To examine  
246 whether *PR1a* expression is caused by the  $\text{Ca}^{2+}$  influx that accompanies wounding, in  
247 addition to viral RSS, we infiltrated leaves of transgenic tobacco plants expressing 2b or  
248 HC-Pro with a  $\text{Ca}^{2+}$  ionophore, A23187, which causes external  $\text{Ca}^{2+}$  influx and thus  
249 elevates intracellular  $\text{Ca}^{2+}$  levels by increasing its ability to cross biological membranes.

250 At 24 h after infiltration with A23187, *PR1a* was induced in transgenic tobacco  
251 plants expressing 2b or HC-Pro but not in transgenic tobacco plants expressing CMV  
252 CP or in wild-type tobacco plants (Fig. 6A). We confirmed that the *PR1a* expression  
253 was not due to a side effect of A23187: infiltration of A23187 did not cause cell death or  
254 other obvious morphological changes in these plant leaves (Fig. 6B), and concurrent  
255 treatment with ethylene glycol-bis( $\beta$ -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid  
256 (EGTA), which chelates  $\text{Ca}^{2+}$ , and A23187 antagonized *PR1a* expression (Fig. 6Ci). We  
257 note that *PR1a* was slightly induced in wild-type plants with A23187 infiltration (Fig.  
258 6Cii). However, this slight *PR1a* induction seems to be qualitatively different from that  
259 induced by viral RSSs and  $\text{Ca}^{2+}$  influx, because the *PR1a* mRNA levels that were  
260 increased by  $\text{Ca}^{2+}$  in 2b-expressing plants were reduced in the presence of EGTA,

261 whereas the *PR1a* levels induced by  $\text{Ca}^{2+}$  in wild-type plants treated with A23187 did  
262 not change in the presence of EGTA. We conclude that the expression of an RSS  
263 together with  $\text{Ca}^{2+}$  influx induces salicylic acid signaling but that neither RSS  
264 expression nor  $\text{Ca}^{2+}$  influx alone is sufficient.  $\text{Ca}^{2+}$  influx induced rgs-CaM expression  
265 (Fig. 6Ci, ii), consistent with our hypothesis that *PR1a* is induced via rgs-CaM. To test  
266 this further, we used a PVX vector that expresses the *rgs-CaM* mRNA sequence without  
267 its initiation codon to knock down the expression of endogenous rgs-CaM by VIGS  
268 [VIGS(*rgs-CaM*)]. When RSS-expressing tobacco plants were inoculated with the PVX  
269 empty vector, *PR1a* expression was induced even without A23187 treatment (Fig. 7A).  
270 We also found induction of *PR1a* in the empty-vector-infected wild-type tobacco plants  
271 treated with A23187. *PR1a* induction by infection of RSS-expressed plants with PVX  
272 without A23187 or by infiltration of PVX-infected tobacco leaves with A23187 is  
273 apparently discrepant to our hypothesis shown in Fig. 5A and discussed later in the  
274 Discussion section. Including these apparently discrepant cases, the *PR1a* inductions  
275 were reduced by infection with the VIGS(*rgs-CaM*) vector (Fig. 7A), suggesting that  
276 *PR1a* induction depends on rgs-CaM.

277 *PR1a* induction was suppressed when salicylate hydroxylase (*NahG*)-expressing  
278 tobacco plants, in which salicylic acid is converted to catechol and thus salicylic acid  
279 signaling is antagonized, were inoculated with the PVX empty vector or CMV $\Delta$ 2b and  
280 then treated with A23187. These results indicate that salicylic acid signaling was  
281 induced in wild-type tobacco plants infected with either the empty PVX vector or  
282 CMV $\Delta$ 2b infection when  $\text{Ca}^{2+}$  influx was artificially induced with A23187 (Fig. 7B).

283

284 **rgs-CaM is necessary for enhanced resistance against CMV in SAR-induced**

285 **tobacco plants**

286 In addition to being an inducer of salicylic acid signaling, we found that rgs-CaM is  
287 involved in salicylic acid–mediated antiviral defense. The inoculation test results in Fig.  
288 4 showed comparable accumulation of CMV CP and genomic RNAs in inoculated and  
289 upper leaves between wild-type and rgs-CaM–knockdown plants, indicating that rgs-  
290 CaM does not interfere with CMV infection. However, when CMV was inoculated into  
291 relatively old tobacco plants (for example, 7 weeks after sowing [Fig. 8Ai]), the rgs-  
292 CaM–knockdown plants developed systemic yellowing of leaves earlier than did the  
293 inoculated wild-type plants. At 16 dpi, CMV could be detected by western blotting only  
294 in non-inoculated upper leaves of inoculated rgs-CaM–knockdown plants (Fig. 8Aii).  
295 The tobacco plants described in Fig. 4 were inoculated at 4 weeks after sowing,  
296 suggesting that the antiviral function of rgs-CaM has two phases: it is dormant in  
297 normally growing young tobacco plants around 4 weeks after sowing but becomes  
298 activated by 7 weeks after sowing.

299         What, then, is different between tobacco plants at 4 and 7 weeks after sowing  
300 that brings about the phase change of the antiviral function of rgs-CaM? A previous  
301 study showed that tobacco plants gradually accumulate salicylic acid during the 7 to 10  
302 weeks after sowing and develop enhanced resistance against tobacco mosaic virus,  
303 probably because of the accumulated salicylic acid (43). Similar age- and salicylic acid-  
304 related resistance against CMV has been reported previously (44, 45). These studies  
305 prompted us to examine whether salicylic acid signaling affects rgs-CaM function by  
306 using a salicylic acid analog, benzo-(1,2,3)-thiadiazole-7-carbothioic acid S-methyl  
307 ester (BTH), which is a strong inducer of SAR via systemic induction of salicylic acid  
308 signaling (46, 47). Systemic symptom expression in leaves was delayed (Fig. 8Bi) and

309 CMV accumulation was drastically reduced in BTH-treated wild-type tobacco plants  
310 relative to the untreated control (Fig. 8Bii, iii), confirming the enhancement of antiviral  
311 resistance by induction of SAR with BTH, as reported previously (48, 49). These effects  
312 were weakened in the rgs-CaM-knockdown plants, indicating that the enhanced  
313 resistance to CMV induced by BTH depends on rgs-CaM (Fig. 8Bii, iii). Judging by the  
314 symptoms observed (Fig. 8Bi) and the results of western blotting with samples of  
315 inoculated leaves (Fig. 8Bii), some resistance was still induced in BTH-treated rgs-  
316 CaM-knockdown plants. This resistance might have been caused by the residual rgs-  
317 CaM in the knockdown plants or by a salicylic acid-mediated defense system that  
318 operates independently but in parallel to the rgs-CaM-mediated defense mechanism. To  
319 examine whether tobacco plants have salicylic acid-mediated defense system(s), which  
320 is not linked to the rgs-CaM-mediated defense mechanism, we conducted similar  
321 experiments using CMV $\Delta$ 2b and PVX because these viruses were considered to lack an  
322 RSS that interact with rgs-CaM. When CMV $\Delta$ 2b was inoculated into wild-type tobacco  
323 plants, CMV $\Delta$ 2b accumulation was drastically reduced by BTH-treatment even in rgs-  
324 CaM-knockdown plants (Fig. 8C), indicating the existence of independent salicylic  
325 acid-mediated defense system(s) that effectively inhibit CMV infection. When PVX  
326 was inoculated into wild-type tobacco plants in which SAR was induced by  
327 pretreatment with BTH, PVX CP accumulated in inoculated and upper leaves, although  
328 to a slightly lesser extent than in non-induced leaves (Fig. 8D). Similar results were  
329 obtained using the rgs-CaM-knockdown tobacco plants. Thus, the SAR induced by  
330 BTH was relatively ineffective against PVX, compared with that against CMV-Y and  
331 CMV $\Delta$ 2b, and we could not conclude whether rgs-CaM contributes to the low level of  
332 SAR against PVX.

333

334 **Reduced accumulation of viral RSSs in SAR-induced transgenic tobacco cells and**  
335 **plants**

336 We previously demonstrated that rgs-CaM binds to and directs degradation of viral  
337 RSSs, CMV 2b and CIYVV HC-Pro, via autophagy (32). The prerequisite of rgs-CaM  
338 for enhanced resistance against CMV but not against CMV $\Delta$ 2b in SAR-induced plants  
339 implies that the rgs-CaM-mediated degradation of viral RSSs might be activated in the  
340 SAR-induced plants. Using cultured transgenic tobacco BY2 cells that constitutively  
341 express CMV 2b, we examined whether the degradation of 2b is activated by SAR  
342 induction. The 2b protein was detected in nuclei in untreated cells by  
343 immunofluorescent staining, but the fluorescent signal disappeared 1 h after BTH  
344 treatment (Fig. 9). The fluorescent signal was, however, retained in cells treated with  
345 both BTH and an autophagy inhibitor (either E64d or concanamycin A), suggesting that  
346 the degradation of 2b, probably via autophagy, was activated by SAR induction, which  
347 leads to resistance against CMV-Y infection.

348 We then examined the effect of Ca<sup>2+</sup> influx on accumulation of the HC-Pro  
349 protein in SAR-induced HC-Pro transgenic tobacco plants because Ca<sup>2+</sup> influx is  
350 expected as a result of wounding during virus infection, as illustrated in Fig. 5A–C.  
351 A23187 treatment reduced accumulation of the HC-Pro protein in SAR-induced HC-Pro  
352 tobacco plants (Fig. 10A). However, A23187 treatment had little effect on accumulation  
353 of the HC-Pro protein in HC-Pro tobacco plants in which SAR was not induced,  
354 suggesting that HC-Pro expression is specifically inhibited in the initial virus-infected  
355 cells of SAR-induced tobacco plants. The upper band (around 25 kDa) of the rgs-CaM  
356 protein extracted from A23187-infiltrated leaf tissue of SAR-induced plants migrated a



357 little more slowly in SDS-PAGE than that extracted from A23187-infiltrated leaf tissue  
358 of non-induced plants (Fig. 10A, right panel, blue arrowheads), implying a change in  
359 the rgs-CaM protein state as a result of SAR induction.

360

## 361 **DISCUSSION**

362 This study revealed that a novel class of protein, calmodulin-like protein rgs-CaM,  
363 functions as an immune receptor for CMV infection and induces salicylic acid  
364 signaling, which is characteristic of immune responses against biotrophic pathogens,  
365 including viruses (8), and is required for SAR induction (2, 6). As mentioned in the  
366 Introduction, the known immune receptors for pathogens in plants are mostly RLKs and  
367 NB-LRRs. RLKs perceive molecules that are conserved among pathogenic  
368 microorganisms but are not found in host plants (pathogen- or microorganism-  
369 associated molecular patterns [PAMPs or MAMPs]) and induce pattern-triggered  
370 immunity (PTI). Host-adapted pathogens develop effector molecules that suppress PTI  
371 and enable their colonization of plants. Another class of receptors, NB-LRRs,  
372 counteractively recognize pathogen effector proteins and induce strong defense  
373 reactions, called hypersensitive responses; this mechanism is termed effector-triggered  
374 immunity (ETI) (50, 51). Several NB-LRRs that perceive virus invasion and induce ETI  
375 have been identified (52), and recent studies of *Arabidopsis* RLKs (53, 54) suggests the  
376 existence of an immune receptor that perceives dsRNAs or other viral factors as viral  
377 PAMPs and induces PTI. In animals, Toll-interleukin 1-like receptors (TLRs), which are  
378 structurally similar to plant RLKs and NB-LRRs, perceive viral RNA and DNA in  
379 endosomes and on cell membranes (55). In addition, RIG-I and MDA5 for viral RNA  
380 and IFI16 and cGAS for viral DNA have been identified as receptors that perceive

381 PAMPs in the cytoplasm and nucleus (56). A NOD-like receptor and other host factors  
382 have been implicated in recognition of viral infection (56). However, no CaM or CaM-  
383 like protein (CML) has previously been identified to be an immune receptor.

384 Plant CaMs and CMLs are  $\text{Ca}^{2+}$  sensors that play important roles in development  
385 and stress responses (57, 58). An increase in the  $\text{Ca}^{2+}$  concentration in the cytoplasm is  
386 one of the earliest events following exposure to environmental stresses and  $\text{Ca}^{2+}$  is a  
387 crucial secondary messenger in the perception of these stresses. In plants, CaMs and  
388 CMLs constitute a relatively large family of  $\text{Ca}^{2+}$  sensor genes along with two other  
389 classes of proteins, calcineurin B-like proteins and  $\text{Ca}^{2+}$ -dependent protein kinases (59).  
390 CaMs and CMLs bind a number of endogenous factors and have no obvious functional  
391 domains except for 1–7 EF-hand motifs for binding  $\text{Ca}^{2+}$ , and thus are considered to  
392 transduce  $\text{Ca}^{2+}$  signals by modifying the activity or conformation of their binding  
393 endogenous proteins (58). rgs-CaM, one of the tobacco CMLs, uniquely binds to  
394 exogenous proteins, diverse viral RSSs [including potyvirus HC-Pro, CMV, (the related)  
395 tomato aspermy virus 2b and human immunodeficiency virus TAT], presumably via  
396 affinity to their positively charged dsRNA-binding sites (28, 32), though there is no  
397 conserved amino acid sequence motif among these dsRNA-binding domains. CaMs and  
398 CMLs are hub proteins, which bind to various substrate proteins through their relatively  
399 disordered binding sites (60). Homology modeling (32, 42) implies that rgs-CaM has a  
400 negatively charged disordered binding site for substrates, which is probably where rgs-  
401 CaM binds diverse viral RSSs. Since viral RSSs are considered to be effectors that  
402 suppress an antiviral PTI-like basal defense (RNA silencing), rgs-CaM is another class  
403 of receptor for viral effectors in addition to NB-LRRs. rgs-CaM perceives not only viral  
404 RSSs but also  $\text{Ca}^{2+}$  cues that induce salicylic acid signaling (Figs. 4 to 7). A recent

405 structural and thermodynamic study by Makiyama et al. (42) revealed that rgs-CaM  
406 binds  $\text{Ca}^{2+}$  at three EF-hand motifs and suggested that  $\text{Ca}^{2+}$  binding at the two EF hands  
407 that show higher affinity to  $\text{Ca}^{2+}$  alters the conformation of rgs-CaM such that the  
408 negatively charged binding sites are more exposed. This supports our model that  
409 salicylic acid signaling is induced by the dual perception of viral RSS and  $\text{Ca}^{2+}$  by rgs-  
410 CaM (Figs. 5A to C and 10B). We assume that the dual perception of viral RSS and  
411  $\text{Ca}^{2+}$  by rgs-CaM avoids nonspecific induction of salicylic acid signaling. Consistently,  
412 overexpression and ectopic expression of rgs-CaM did not always induce defense  
413 responses and salicylic acid signaling (Fig. 1). Because plant cells are surrounded by a  
414 cell wall, virus invasion seems to require mechanical wounding, which would cause  
415  $\text{Ca}^{2+}$  influx in the virus-invaded cells. The normal mechanism of CMV infection in the  
416 field is via aphid feeding and aphid feeding has been reported to cause  $\text{Ca}^{2+}$  influx in  
417 tobacco plants (61, 62). In general, defense responses against various abiotic and biotic  
418 stress responses involve  $\text{Ca}^{2+}$  fluxes (63), and virus infection is known to lead to an  
419 increase of the cytoplasmic  $\text{Ca}^{2+}$  concentration (64). We assume this is why *PR1a* was  
420 induced in PVX-infected transgenic tobacco plants expressing viral RSSs without  
421 artificial  $\text{Ca}^{2+}$  influx induced by A23187 (Fig. 7A). Therefore, the dual perception of a  
422 viral component and  $\text{Ca}^{2+}$  seems suitable as a viral infection cue to specifically induce  
423 immune responses. One drawback to recognition of a viral RSS as an infection cue is  
424 that it is incapable of immediate induction of immune responses because most viral  
425 RSSs, including 2b and HC-Pro, are not included in the invading virion, but are  
426 expressed during establishment of viral infection and viral multiplication. As described  
427 below, the rgs-CaM-induced immune responses do not appear to prevent primary virus  
428 infection; rather, salicylic acid signaling among them may contribute to prevent

429 subsequent infection by viruses possessing RSSs that interact with rgs-CaM via its  
430 autoactivation in SAR-induced plants. Therefore, the rgs-CaM-induced immune  
431 responses do not necessarily need to be induced immediately. In the present study, the  
432 induction of rgs-CaM-mediated salicylic acid signaling after wounding of transgenic  
433 plants expressing viral RSSs took 24 h (Fig. 5E), which is slower than that seen with  
434 ETI (hypersensitive response) (65).

435 rgs-CaM may have the ability to induce salicylic acid signaling in response to  
436 viral or host proteins other than viral RSS. Under natural conditions, rgs-CaM does not  
437 seem to be involved in induction of salicylic acid signaling in response to PVX and  
438 CMV $\Delta$ 2b infection (Figs. 3 and 4). However, when Ca<sup>2+</sup> influx was artificially induced  
439 with A23187 in wild-type plants, salicylic acid signaling was induced by infection with  
440 either PVX or CMV $\Delta$ 2b (Fig. 7), and salicylic acid signaling induced by PVX in the  
441 presence of Ca<sup>2+</sup> was dependent on rgs-CaM (Fig. 7A). The triple gene block protein1  
442 (TGBp1) of PVX is an RSS. The suppression mechanism of RNA silencing by TGBp1  
443 is not through binding to dsRNA; instead, TGBp1 was reported to bind to AGO1–  
444 AGO4 and lead to degradation of AGO1 via the 26S proteasome (66). Considering that  
445 rgs-CaM probably binds to the dsRNA binding sites of viral RSSs, rgs-CaM may not  
446 bind TGBp1. More strikingly, tobacco plants must be able to recognize CMV proteins  
447 other than its RSS (2b) for there to be induction of salicylic acid signaling by CMV $\Delta$ 2b  
448 (Fig. 7B). At first glance, the results in Fig. 7 seem to contradict our conclusion that rgs-  
449 CaM perceives viral RSSs and Ca<sup>2+</sup> as virus infection cues to induce salicylic acid  
450 signaling. One possible explanation is that rgs-CaM may have weak affinity to PVX and  
451 CMV protein(s) other than 2b, and can bind to them when Ca<sup>2+</sup> influx is stimulated by  
452 A23187 infiltration (Fig. 10B, right panel). The substrate (RSS) binding domain of rgs-

453 CaM was predicted to be more exposed when Ca<sup>2+</sup> binds to rgs-CaM at its EF hands  
454 (42). Therefore, under specific conditions, such as when wild-type tobacco leaves that  
455 were infected with PVX or CMV Δ 2b were subsequently infiltrated with A23187, rgs-  
456 CaM may perceive other PVX and CMV protein(s) to induce salicylic acid signaling.  
457 Another possibility is simply that rgs-CaM binds to host intermediate(s) that is induced  
458 by virus infection for salicylic acid signaling.

459 RNA silencing and salicylic acid-mediated immunity are two major antiviral  
460 systems in plants and their linkage has been suggested (18-26). The present study also  
461 revealed a link between RNA silencing and salicylic acid-mediated immunity via a  
462 single host factor, rgs-CaM, which suppresses antiviral RNA silencing as an  
463 endogenous RSS but induces salicylic acid signaling by perceiving viral RSS as an  
464 immune receptor (e.g., in the case of CMV). Pruss et al. (67) reported that transgenic  
465 tobacco plants expressing HC-Pro show enhanced resistance to both heterologous  
466 viruses that have different RSSs and fungal pathogens; depending on the pathogen,  
467 resistance could be either salicylic acid-dependent or -independent. The mechanism  
468 underlying this viral RSS-induced enhanced resistance against multiple pathogens  
469 remains unclear. In those transgenic plants (68), rgs-CaM could induce salicylic acid  
470 signaling in response to Ca<sup>2+</sup> influx caused by infection with pathogens and thus partly  
471 contribute to the enhanced resistance in a salicylic acid-dependent manner.

472 Another significant observation of this study is uncovering a part of the  
473 molecular mechanism underlying the enhanced resistance against a virus in SAR-  
474 induced plants. We previously reported the antiviral function of rgs-CaM (32). The  
475 present study revealed that this antiviral function is not constitutively active but exhibits

476 a phase change from dormant to activated after SAR induction via salicylic acid  
477 signaling (Figs. 4, 8–10). We previously showed that, without artificial induction of  
478 SAR, the rgs-CaM–overexpressing transgenic tobacco plants (rgs-CaM16) inhibit CMV  
479 infection (32). However, this is not contradictory to the present study because  
480 overexpression of rgs-CaM induces salicylic acid signaling systemically in this  
481 transgenic line (Fig. 1) and thus induces SAR. Since CMV infection has been reported  
482 to induce salicylic acid signaling in this study (Fig. 4) and previously (44, 68, 69), one  
483 may expect that rgs-CaM autoactivates its antiviral function for SAR during CMV  
484 infection via its perception of CMV 2b. However, rgs-CaM did not effectively inhibit  
485 CMV infection in relatively young plants (Fig. 4) though it did in older plants (Fig. 8A).  
486 CMV 2b has been reported to interfere with salicylic acid and jasmonic acid signaling  
487 (44, 68, 69).  $\text{Ca}^{2+}$  influx induced by A23187 caused rgs-CaM protein accumulation in  
488 both wild-type and 2b-expressing transgenic plants (Fig. 6C). However, its  
489 accumulation level was lower in 2b-expressing plants, in which *PR1a* was induced, than  
490 in wild-type tobacco plants. Our previous study (32) suggested that both rgs-CaM and  
491 viral RSS proteins are posttranslationally regulated via the 26S proteasome and  
492 autophagy and that rgs-CaM directs degradation of these RSS proteins. The rgs-CaM–  
493 mediated degradation of viral RSS proteins, was enhanced by salicylic acid signaling  
494 (Figs. 8 – 10). Overexpression of *rgs-CaM* did not always result in increased  
495 accumulation of rgs-CaM protein, induction of salicylic acid signaling, and other  
496 defense responses (Fig. 1), suggesting complex interactions (counteraction or  
497 neutralization) among rgs-CaM, 2b, salicylic acid signaling and protein degradation  
498 pathways.

499           It is generally assumed that plants and animals inhibit infection by any  
500 pathogens to reduce the threat of disease. However, this and previous studies have  
501 shown biased reactions of tobacco plants against pathogenic viruses via the antagonistic  
502 functions of rgs-CaM. rgs-CaM was initially shown to be an endogenous RSS by using  
503 transgenic *N. benthamiana* in which the tobacco *rgs-CaM* gene was overexpressed by  
504 the CaMV 35S promoter (28). In that study, the overexpressed tobacco *rgs-CaM*  
505 interfered with VIGS of GFP by a PVX vector, resulting in increased fluorescence and  
506 accumulation of the GFP transgene and the PVX genomic RNA itself (28). Li et al. (34)  
507 reported that infection by tomato yellow leaf curl China virus, a member of the genus  
508 *Begomovirus*, was facilitated or inhibited in transgenic *N. benthamiana* plants in which  
509 rgs-CaM was overexpressed or silenced, respectively. They also confirmed the RSS  
510 activity of rgs-CaM (34, 70). Additionally, infection by tomato golden mosaic virus,  
511 another member of the genus *Begomovirus*, was shown to be facilitated in transgenic  
512 *Arabidopsis* plants in which *Arabidopsis* CML39, one of the proteins most similar to  
513 rgs-CaM among 50 *Arabidopsis* CMLs, was overexpressed (35). Taken together with  
514 data in this study, in normally growing plants, rgs-CaM facilitates infection by members  
515 of the genus *Begomovirus*, but not CMV (*Cucumovirus*) and PVX (*Potexvirus*),  
516 probably by its RSS activity, but inhibits CMV infection by its phase-changed antiviral  
517 activity that directs degradation of CMV 2b via autophagy after SAR induction.

518           Constitutive activation of plant immune systems results in inhibition of plant  
519 growth (71), as also shown here by overexpression of rgs-CaM (Fig. 1). This trade-off  
520 between immunity and growth in plants has driven the evolution of immune receptors  
521 for recognition of pathogen invasion that effectively induce defense mechanisms only  
522 when needed. The receptor and conditional effector functions of rgs-CaM (that is, its

523 phase change via SAR induction) suggest that tobacco changes its reaction to viral  
524 infection according to environmental conditions via rgs-CaM. rgs-CaM strongly inhibits  
525 infection by viruses that express RSSs that directly interact with it, such as CMV, only  
526 under environmental conditions with a high frequency of infection by pathogens, which  
527 leads to SAR induction (Fig. 10B, left and center panels). In general, viral RSSs  
528 function as virulence factors not only by enhancing virus multiplication that leads to  
529 increased expression of other viral virulence factors via suppressing antiviral RNA  
530 silencing, but also by disrupting host gene expression controlled by the small-RNA  
531 pathways in infected cells. This biased and conditional antiviral defense system has  
532 presumably developed as a means of counteracting RSS-expressing virulent viruses to  
533 avoid the cost of constitutive defense activation while reducing the damage from the  
534 virus infection.

535

## 536 **MATERIALS AND METHODS**

### 537 **PVX vectors carrying rgs-CaM cDNA and expression cassettes**

538 The *rgs-CaM* ORF and the ORF lacking its initial codon were cloned between the *Clai*  
539 and *SalI* sites of the PVX vector pPC2S (72) to generate PVX-rgs-CaM and PVX-rgs-  
540 CaM(-atg) [VIGS(*rgs-CaM*)], respectively. After linearization of these plasmids by  
541 digestion with *SpeI*, infectious RNAs were transcribed by T7 RNA polymerase with the  
542 7-methylguanosine-5'-phosphate cap analog (Thermo Fisher Scientific Inc., Waltham,  
543 MA, USA) from the linearized plasmids and used as inocula for mechanical inoculation.  
544 The *rgs-CaM* ORFs with/without the initiation codon were also cloned between the  
545 *XbaI* and *SacI* sites of pE2113 (73) and the cloned plasmids, pE2113-rgs-CaM and



546 pE2113-rgs-CaM(-atg), were used for transfection of tobacco protoplasts to express rgs-  
547 CaM under control of the CaMV 35S promoter.

548

#### 549 **Transgenic tobacco plants and virus inoculation**

550 Transgenic tobacco plants (*N. tabacum* cv. Xanthi), in which rgs-CaM was either  
551 overexpressed or knocked down, were made previously (32). Transgenic tobacco plants  
552 (*N. tabacum* cv. BY4) expressing viral RSSs were also made previously (32).

553 Transgenic tobacco plants expressing CMV CP and NahG were made similarly to those  
554 expressing viral RSSs (32). T2 or later generations of transgenic tobacco plants, all of  
555 which were shown to be kanamycin resistant, were grown under a 16-h light/8-h dark  
556 photoperiod at 25°C for virus inoculation and other experiments. *N. benthamiana* leaves  
557 infected with CMV-Y; CMV $\Delta$ 2b, which lacked 2b and was designated CMV-H1 in a  
558 previous study (74); and the PVX vectors were used as inocula for mechanical  
559 inoculation with Carborundum and stored in a deep freezer at -80°C until needed.

560

#### 561 **BTH and Ca<sup>2+</sup> ionophore treatment**

562 A salicylic acid analog, BTH, was spread on tobacco leaves with cotton tufts that were  
563 dipped in 1 mM BTH, 1.4% (vol/vol) acetone as a solvent, and 0.2% Tween-20.

564 Phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM

565 Na<sub>2</sub>HPO<sub>4</sub>·12H<sub>2</sub>O, and 2 mM KH<sub>2</sub>PO<sub>4</sub> pH 7.4) containing 75  $\mu$ M of Ca<sup>2+</sup> ionophore

566 A23187 (MilliporeSigma, St. Louis, MO, USA) was prepared by diluting A23187 stock  
567 solution (5 mg/ml of A23187 dissolved in DMSO) with PBS, and the diluted A23187

568 solution with/without 10 mM EGTA was infiltrated into leaves with a syringe.

569

570 **Preparation, transfection, and assays of tobacco protoplasts**

571 Tobacco mesophyll protoplasts were prepared from wild-type tobacco plants (*N.*  
572 *tabacum* cv. Xanthi) and transfected with pE2113 vectors as described previously (75).  
573 Assays following transfection were also carried out according to the method from the  
574 previous study (75). H<sub>2</sub>O<sub>2</sub> signals, indicative of ROS generation, were visualized with  
575 500 nM 2',7'-dichlorofluorescein-diacetate (H<sub>2</sub>DCF) (MilliporeSigma) 5 h after  
576 transfection. The images were observed with a fluorescence microscope (Leica DMI  
577 6000B; Leica, Tokyo) and H<sub>2</sub>DCF signals were visualized with excitation at 488 nm  
578 (emission: 498 to 532 nm). Eleven hours after transfection, protoplasts were exposed to  
579 0.04% Evans blue dye (an indicator of cell death) for 5 min and then observed with  
580 light microscopy (Olympus BX51; Olympus, Tokyo).

581

582 **RT-PCR, semi-quantitative RT-PCR, real-time RT-PCR, and northern blotting**

583 After tobacco leaves were ground in liquid nitrogen, total RNA was extracted using the  
584 TRIzol reagent according to the manufacturer's manual (Thermo Fisher Scientific).  
585 Each RNA sample was treated with RNase-free DNase I (Roche Diagnostics, Basel,  
586 Switzerland). First-strand cDNAs were synthesized from 1 µg of RNA extracts by a  
587 modified M-MLV reverse transcriptase, ReverTra Ace (Toyobo, Osaka, Japan).  
588 Accumulation of viral genomic RNAs and endogenous mRNAs was detected by PCR in  
589 a mixture (25 µl) containing cDNAs corresponding to 0.05 µg RNA, 0.4 µM of each of  
590 the specific primer pairs listed in Table 1, 0.2 mM dNTP, and 0.625 U Ex Taq DNA  
591 polymerase (TaKaRa, Otsu, Japan). PCR mixtures for *PR1a* were incubated for 2 min at  
592 94°C, followed by 28 cycles of 94°C for 30 s, 62°C for 30 s, and 72°C for 40 s, and  
593 PCR products were fractionated with 2% agarose gel electrophoresis. Semi-quantitative

594 RT-PCR was done for *rgs-CaM* by using 24 cycles of 94°C for 30 s, 59°C for 30 s, and  
595 72°C for 30 s, and for 18S rRNA by using 15 cycles of 94°C for 30 s, 58°C for 30 s,  
596 and 72°C for 30 s. Real-time PCR was performed by using the DNA Engine Opticon 2  
597 system (Bio-Rad Laboratories, Hercules, CA, USA) according to the method in a  
598 previous study (76). The reaction mixture (25 µl) contained 0.625 U of Ex Taq  
599 (TaKaRa), Ex Taq buffer, 0.2 mM dNTP, 0.2 µM (each) forward and reverse primers  
600 listed in Table 1, SYBR Green (30,000 × dilution) (Thermo Fisher Scientific), and  
601 cDNA corresponding to 12.5 ng of total RNA. Samples were incubated for 2 min at  
602 95°C, followed by 39 cycles of 95°C for 10 s, 58°C for *rgs-CaM* or 59°C for *PR1a* for  
603 20 s, and 72°C for 20 s. Northern blotting was performed as described previously (77)  
604 using DIG-labeled cRNA probes (Roche Diagnostics). These probes were made from  
605 the target mRNA sequences, PVX genomic RNA sequence, and the conserved  
606 nucleotide sequence at the 3'-terminal regions of CMV genome segments using the  
607 primers listed in Table 1. RNA samples (2–5 µg) were fractionated by denaturing  
608 agarose gel electrophoresis and transferred onto a nylon membrane (Hybond-N; GE  
609 Healthcare, Chicago, IL, USA). Chemiluminescence signals were quantitatively  
610 detected by a LAS-4000 mini PR Lumino-image analyzer (GE Healthcare).

611

### 612 **Western blotting**

613 Western blotting was carried out as described previously (32). Tobacco leaf tissues were  
614 homogenized in liquid nitrogen and then dissolved in 12-fold (volume/mass) urea-  
615 denaturing buffer containing 4.5 M urea, 1% (vol/vol) Triton X-100, 0.5% DTT, 0.0625  
616 M Tris-HCl pH 6.8, 2% (wt/vol) SDS, 5% mercaptoethanol, 5% sucrose, and 0.002%

617 bromophenol blue. The extracts were centrifuged to collect the supernatants. Equal  
618 amounts of samples were separated by 10% SDS/PAGE. Fractionated proteins were  
619 then transferred to Immobilon PVDF membranes (MilliporeSigma), and the blots were  
620 probed with anti-PVX CP, anti-CMV CP, anti-2b, and anti-rgs-CaM rabbit polyclonal  
621 antibodies. Proteins were visualized using antirabbit secondary antibodies conjugated to  
622 alkaline phosphatase, followed by treatment with CDP-Star solutions (Roche  
623 Diagnostics, Basel, Switzerland) for chemiluminescence detection. Chemiluminescent  
624 signals were quantitatively detected by a LAS-4000 mini PR Lumino-image analyzer  
625 (GE Healthcare).

626

#### 627 **Immunohistochemical studies with tobacco BY2 cultured cells**

628 Tobacco BY2 cultured cells were transformed with the CMV 2b gene under the control  
629 of the CaMV 35S promoter in a previous study (78), in which the transformed BY2 was  
630 called cell line Y2b–BY2. Transgenic BY2 cells expressing 2b were pretreated with 10  
631  $\mu\text{M}$  BTH with/without autophagy inhibitors E64d (10  $\mu\text{M}$ ) and concanamycin A (0.1  
632  $\mu\text{M}$ ) for 1 h and then assayed for endogenous rgs-CaM and CMV 2b as described  
633 previously (32). The fixed cells were immunofluorescently stained with their specific  
634 primary and CF594 goat antirabbit IgG secondary antibodies (Biotium, Fremont, CA,  
635 USA). These cells were also fluorescently stained with 4',6-diamino-2-phenylindole  
636 (DAPI) to detect nuclei. Photomicrographs were taken using a Leica DMI6000 B  
637 microscope (Leica Microsystems). Image colors were then reassigned using AF6000  
638 ver. 1.5 software.

639

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646

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899

## 900 **Figure Legends**

901 **FIG 1** Overexpressed and ectopically expressed rgs-CaM elicits immune responses in  
902 tobacco, implying a link between rgs-CaM and salicylic acid signaling. (Ai) Transgenic  
903 tobacco plants overexpressing rgs-CaM showed phenotypic characteristics indicating  
904 activation of immune responses, such as necrosis and dwarfing, at 7 weeks after sowing  
905 of transgenic lines 16 (rgs-CaM16) and 23 (rgs-CaM23). (Aii) Within each of these two  
906 transgenic lines, severity of the lesion mimic phenotype was variable. Individual plants  
907 from each line are shown in order from mild (1) to severe (6) phenotypes. These  
908 individuals were confirmed to have the rgs-CaM transgene by detecting the 35S and  
909 rgs-CaM nucleotide sequences by PCR (Aiii). PCR products amplified from the binary  
910 vector pBE2113-rgs-CaM, with which tobacco plants were transformed, with the same  
911 primer pairs were loaded as a control (lane C). Cell death (B) and generation of reactive  
912 oxygen species (ROS) (C) in leaves were compared between transgenic tobacco  
913 overexpressing rgs-CaM and wild-type (WT) by Evans blue and 2',7'-  
914 dichlorofluorescein-diacetate (H<sub>2</sub>DCF) staining, respectively. BF indicates bright-field  
915 images. (Di) Expression of *PR1a*, an indicator of salicylic acid signaling, was

916 investigated by northern blotting. Samples from seven plants of transgenic line 16 were  
917 ordered from left to right in increasing severity of the phenotype. The *PR1a* mRNA  
918 level was investigated by northern blotting. Overexpression of rgs-CaM in these plants  
919 was confirmed by western blotting for its protein and semi-quantitative RT-PCR (sqRT-  
920 PCR) for its mRNA. Wild-type (WT) tobacco was used as a control. (Dii) Transgenic  
921 line 23, which overexpressed rgs-CaM and showed a similar phenotype to line 16, was  
922 also shown by northern blotting to induce *PR1a* expression; as in the case of line 16,  
923 expression varied within the line. Coomassie brilliant blue–stained (CBB) and ethidium  
924 bromide–stained (*rRNA*) gels are shown as loading controls.

925

926 **FIG 2** Defense responses and salicylic acid signaling were induced by transient  
927 expression of rgs-CaM. (A) A PVX vector expressing rgs-CaM (PVX-rgs-CaM), a PVX  
928 vector expressing the subgenomic RNA containing the rgs-CaM open reading frame  
929 without its initiation codon [PVX-rgs-CaM(-atg)], and an empty vector (PVX) were  
930 inoculated into wild-type tobacco (cv. Xanthi) plants. Inoculated leaves at 7 days post-  
931 inoculation are shown. Their *PR1a* expression was investigated by real-time PCR. The  
932 mRNA levels relative to that of mock-inoculated plants are shown in the bar graph ( $n =$   
933 4). Error bars indicate SE. Student's *t* test was applied to the data and \*\* indicates *P*  
934 value of <0.01. (B) Protoplasts prepared from wild-type tobacco plants were transfected  
935 with expression cassettes with the rgs-CaM cDNA and the modified cDNA without the  
936 initiation codon (rgs-CaM(-atg)), and stained with Evans blue. Black bars indicate 50  
937  $\mu\text{m}$ . The cell death rate (Evans blue–stained cells/total cells) is shown in the bar graph  
938 ( $n = 5$ ). Error bars indicate SE. Student's *t* test was applied to the data and \* indicates *P*  
939 value of <0.05 relative to protoplasts without transfection (Cont). (C) When the

940 protoplasts described in (B) were stained with H<sub>2</sub>DCF, protoplasts generating ROS were  
941 detected among those transfected with the rgs-CaM expression cassette. Among  
942 protoplasts transfected with rgs-CaM(-atg) or not transfected (Cont), no H<sub>2</sub>DCF signal  
943 was detected. BF indicates bright-field images. White bars indicate 10 μm.

944

945 **FIG 3** Susceptibility of rgs-CaM-knockdown tobacco plants to PVX, and salicylic acid  
946 signaling in response to PVX infection. (A) PVX was inoculated into rgs-CaM-  
947 knockdown (IR-rgs-CaM) and wild-type (WT) tobacco plants. Accumulation of PVX  
948 CP and rgs-CaM and of PVX genomic and subgenomic RNAs (*gPVX* and *sgPVX*,  
949 respectively) was investigated in the inoculated leaves by western and northern blotting,  
950 respectively, at 1 and 3 days post-inoculation (dpi). (B) The same type of inoculation as  
951 in (A) was done with more individual plants ( $n = 8$ ). Accumulation of PVX genomic  
952 RNA was measured by real-time PCR using a pair of primers for amplification of a  
953 partial cDNA sequence of viral RNA-dependent RNA polymerase (RdRp). Similarly,  
954 accumulation of PVX RNAs including both genomic and subgenomic RNAs was  
955 measured with a pair of primers for amplification of a partial cDNA of viral coat protein  
956 (CP). The levels of *PR1a* (C) and *rgs-CaM* (D) mRNA were investigated by real-time  
957 PCR ( $n = 5$ ). mRNA levels relative to those of mock-inoculated plants are shown. Bars  
958 indicate SE. Student's *t* test was applied to the data and \* indicates *P* values of <0.05.  
959 Coomassie brilliant blue-stained (CBB) and ethidium bromide-stained (*rRNA*) gels are  
960 shown as loading controls of western and northern blotting, respectively.

961

962 **FIG 4** Implication of rgs-CaM involvement in salicylic acid signaling in response to  
963 infection by CMV. CMV-Y (A and B) and CMV lacking 2b (CMVΔ2b) (C and D) were

964 inoculated into wild-type (WT) and rgs-CaM–knockdown (IR-rgs-CaM) tobacco plants  
965 and accumulation of CMV CP, 2b and rgs-CaM proteins, CMV genomic and  
966 subgenomic RNAs (*gCMV* and *sgCMV*), respectively (A and C), and the *PR1a* and *rgs-*  
967 *CaM* mRNAs were investigated ( $n = 3$ ) as done in Fig. 3 (B and D). (B, exp. 2) The  
968 same type of inoculation as in (exp. 1) was done with more individual plants ( $n = 9$ ) and  
969 investigated the *PR1a* mRNA level. Error bars indicate SE. Student's *t* test was applied  
970 to the data and \* and \*\* indicate *P* value of <0.05 and <0.01, respectively. Coomassie  
971 brilliant blue–stained (CBB) and ethidium bromide–stained (*rRNA*) gels are shown as  
972 loading controls.

973

974 **FIG 5** Model of salicylic acid signaling in response to CMV infection in tobacco plants  
975 (A–C) and salicylic acid signaling in response to wounding stress (D, E). (A–C) In this  
976 model, rgs-CaM functions as an immune receptor that perceives viral RSS and  $Ca^{2+}$ .  
977 Tobacco plants induce salicylic acid signaling when rgs-CaM perceives both 2b and  
978  $Ca^{2+}$  as CMV infection cues in an inoculated leaf (A) but not when rgs-CaM perceives  
979 either 2b or  $Ca^{2+}$  alone, e.g., in a non-inoculated upper leaf (B) or in a leaf inoculated  
980 with CMV lacking 2b (CMV $\Delta$ 2b) (C). (D) Transgenic tobacco plants expressing CMV  
981 2b and CIYVV HC-Pro were microperforated by bundled pins. Immediately after  
982 microperforation, cell death (middle panels) and ROS generation (lower panels) were  
983 visualized by staining leaves with Evans blue or  $H_2DCF$ , respectively. (E) Expression of  
984 *PR1a* was investigated by RT-PCR at different time points after microperforation of  
985 tobacco leaves.

986

987 **FIG 6** Induction of salicylic acid signaling in viral RNA silencing suppressor (RSS)-

988 expressing tobacco plants with Ca<sup>2+</sup> influx. (A) A Ca<sup>2+</sup> ionophore, A23187 (75 μM),  
989 was infiltrated into leaves of wild-type (WT) and transgenic tobacco plants expressing  
990 2b, HC-Pro, or CMV CP. At 24 h after infiltration, the mRNA levels of *PR1a* were  
991 investigated by northern blotting. + and – indicate infiltration of phosphate buffer (PBS)  
992 with and without A23187, respectively. (B) Tobacco leaves were infiltrated with  
993 A23187. A23187 was dissolved in PBS at the indicated concentrations and used to  
994 infiltrate wild-type (WT) and transgenic tobacco expressing RNA silencing suppressors  
995 CMV 2b and CIYVV HC-Pro. Photographs were taken 24 h after infiltration with  
996 A23187. (Ci, ii) To test whether *PR1a* induction was dependent on Ca<sup>2+</sup> influx, EGTA  
997 (10 mM) was infiltrated along with A23187. *PR1a* and *rgs-CaM* mRNA levels and *rgs-*  
998 *CaM* protein levels were investigated by northern and western blotting, respectively, 1  
999 and 24 h after infiltration. Coomassie brilliant blue–stained (CBB) and ethidium  
1000 bromide–stained (*rRNA*) gels are shown as loading controls.

1001

1002 **FIG 7** *PR1a* induction depends on *rgs-CaM*. (A) Wild-type (WT) and transgenic  
1003 tobacco expressing RNA silencing suppressors CMV 2b and CIYVV HC-Pro were  
1004 inoculated with a PVX empty vector (PVX) and a PVX vector expressing the *rgs-CaM*  
1005 ORF sequence lacking the initiation codon as a means of inducing VIGS of *rgs-CaM*  
1006 [VIGS(*rgs-CaM*)]. These inoculated leaves were infiltrated with A23187 (+) or buffer  
1007 alone (–), 3 days after inoculation with PVX. The levels of *PR1a* mRNA, PVX CP, and  
1008 *rgs-CaM* mRNA were investigated by northern blotting, western blotting, and semi-  
1009 quantitative RT-PCR, respectively, 24 h after infiltration with A23187. Samples were  
1010 also prepared from plants that were inoculated with buffer but not infiltrated (Mock)  
1011 and those that were neither inoculated nor infiltrated (Cont). (B) WT and transgenic



1012 tobacco plants expressing salicylate hydroxylase (NahG), which antagonizes salicylic  
1013 acid signaling, were inoculated with PVX and CMV $\Delta$ 2b and infiltrated with A23187 at  
1014 3 days postinoculation. The levels of *PRIa* mRNA and viral CPs were investigated by  
1015 northern and western blotting, respectively, 24 h after infiltration with A23187. Samples  
1016 were also prepared from buffer-inoculated plants without infiltration (Mock).  
1017 Coomassie brilliant blue–stained (CBB) and ethidium bromide–stained (*rRNA*) gels are  
1018 shown as loading controls.

1019

1020 **FIG 8** Enhanced resistance against CMV-Y in SAR-induced tobacco plants depends on  
1021 *rgs*-CaM. (Ai) Comparison of symptoms (yellowing) on non-inoculated upper leaves of  
1022 tobacco plants inoculated with CMV-Y. CMV-Y was inoculated into wild-type (WT)  
1023 and *rgs*-CaM–knockdown (IR-*rgs*-CaM) tobacco plants 7 weeks after sowing. The  
1024 photograph was taken at 16 days postinoculation (dpi) with CMV-Y. All of the *rgs*-  
1025 CaM–knockdown tobacco plants that were inoculated with CMV-Y developed systemic  
1026 symptoms on their leaves, but wild-type tobacco plants did not express symptoms. (Aii)  
1027 The difference in susceptibility between wild-type and *rgs*-CaM–knockdown plants was  
1028 confirmed by detecting CMV CP in non-inoculated upper leaves of these plants by  
1029 western blotting. (Aiii) The mRNA level of *rgs*-CaM relative to that of mock-inoculated  
1030 wild-type plants was investigated by real-time PCR and shown in the bar graph ( $n = 3$ ).  
1031 Error bars indicate SE. Student's *t* test was applied to the data and \* indicates *P* value of  
1032  $<0.05$ . (Bi) Five days after SAR induction by treatment with benzo-(1,2,3)-thiadiazole-  
1033 7-carbothioic acid S-methyl ester (BTH), WT and IR-*rgs*-CaM tobacco plants were  
1034 inoculated with CMV-Y. Control plants (Cont) were treated with a solution containing  
1035 1.4% (vol/vol) acetone and 0.2% Tween-20 (the solution used to dissolve BTH).

1036 Symptoms on upper leaves were photographed 30 dpi. (Bii, iii) CMV CP and 2b  
1037 proteins were detected by western blotting. CMV genomic and subgenomic RNAs  
1038 (*gCMV* and *sgCMV*, respectively), *rgs-CaM* and *PR1a* mRNA were detected by  
1039 northern blotting. Coomassie brilliant blue–stained (CBB) and ethidium bromide–  
1040 stained gels are shown as loading controls. (C) Experiments similar to those shown in  
1041 (B) were done with CMV $\Delta$ 2b. (D) PVX CP accumulation in plants inoculated with  
1042 PVX 5 days after BTH treatment. Accumulation of PVX CP was detected in inoculated  
1043 and non-inoculated upper leaves by western blotting. CBB-stained gels are shown as  
1044 loading controls. Control samples were prepared from buffer-inoculated plants (Mock).  
1045

1046 **FIG 9** Degradation of CMV 2b is enhanced by BTH in transgenic BY2 cultured  
1047 tobacco cells expressing 2b. Transgenic BY2 cultured cells expressing 2b were treated  
1048 with benzo-(1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester (BTH) by adding it  
1049 into the medium at a final concentration of 10  $\mu$ M with or without an inhibitor,  
1050 concanamycin A (concaA) at 0.1  $\mu$ M (A) or E64d at 10  $\mu$ M (B). The CMV 2b and *rgs-*  
1051 *CaM* proteins were detected by immune staining using specific fluorescent secondary  
1052 antibodies 1 h after treatment with BTH with or without an inhibitor. Nuclei were  
1053 visualized by DAPI staining. Differential interference contrast (DIC) images are also  
1054 shown. White bars indicate 25  $\mu$ m.

1055

1056 **FIG 10** Reduction of CIYVV HC-Pro accumulation in transgenic tobacco plants  
1057 expressing HC-Pro (A) and schematic models of detection and counteraction of viral  
1058 RSSs by *rgs-CaM* (B). (A, left) Four leaves (1–4) of individual transgenic plants  
1059 expressing HC-Pro were treated with benzo-(1,2,3)-thiadiazole-7-carbothioic acid S-

1060 methyl ester (BTH). A23187 in PBS was infiltrated into one half of a leaf 1 day after  
1061 BTH treatment; the other half was infiltrated with buffer (PBS). (A, right) The HC-Pro  
1062 and rgs-CaM proteins were detected by western blotting. Values under the HC-Pro  
1063 panel were band intensity of samples from the leaf part infiltrated with A23187 relative  
1064 to that without A23187 in the same leaf (1–4). (B, left) In normally growing tobacco  
1065 plants, the rgs-CaM-mediated defense system does not inhibit CMV infection but  
1066 induces salicylic acid (SA) signaling via perception of CMV 2b and  $\text{Ca}^{2+}$  as CMV  
1067 infection cues. (B, center) When the phase of rgs-CaM is changed by SAR induction,  
1068 subsequent CMV infection is inhibited by rgs-CaM-mediated anti-RSS defense  
1069 reactions. rgs-CaM directs degradation of RSS (CMV 2b) via autophagy, resulting in  
1070 reinforcement of antiviral RNA silencing in addition to SA-mediated antiviral  
1071 immunity. (B, right) When plants are infected with PVX or CMV $\Delta$ 2b and  $\text{Ca}^{2+}$  influx is  
1072 artificially induced with A23187, SA signaling is induced, probably via perception by  
1073 rgs-CaM of  $\text{Ca}^{2+}$  and viral proteins other than RSS or host intermediate proteins that are  
1074 induced by virus infection.  
1075

Figure 1

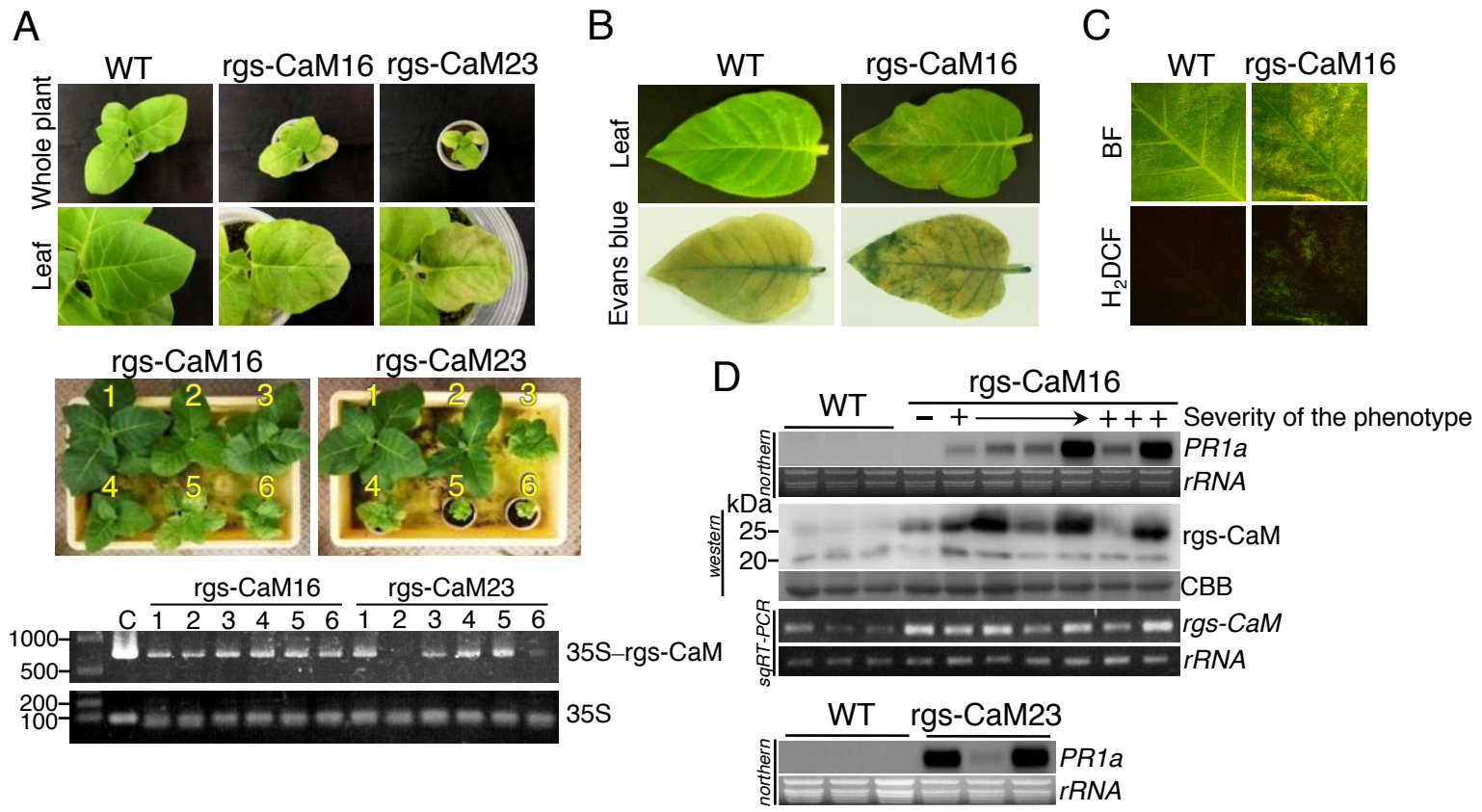


Figure 2

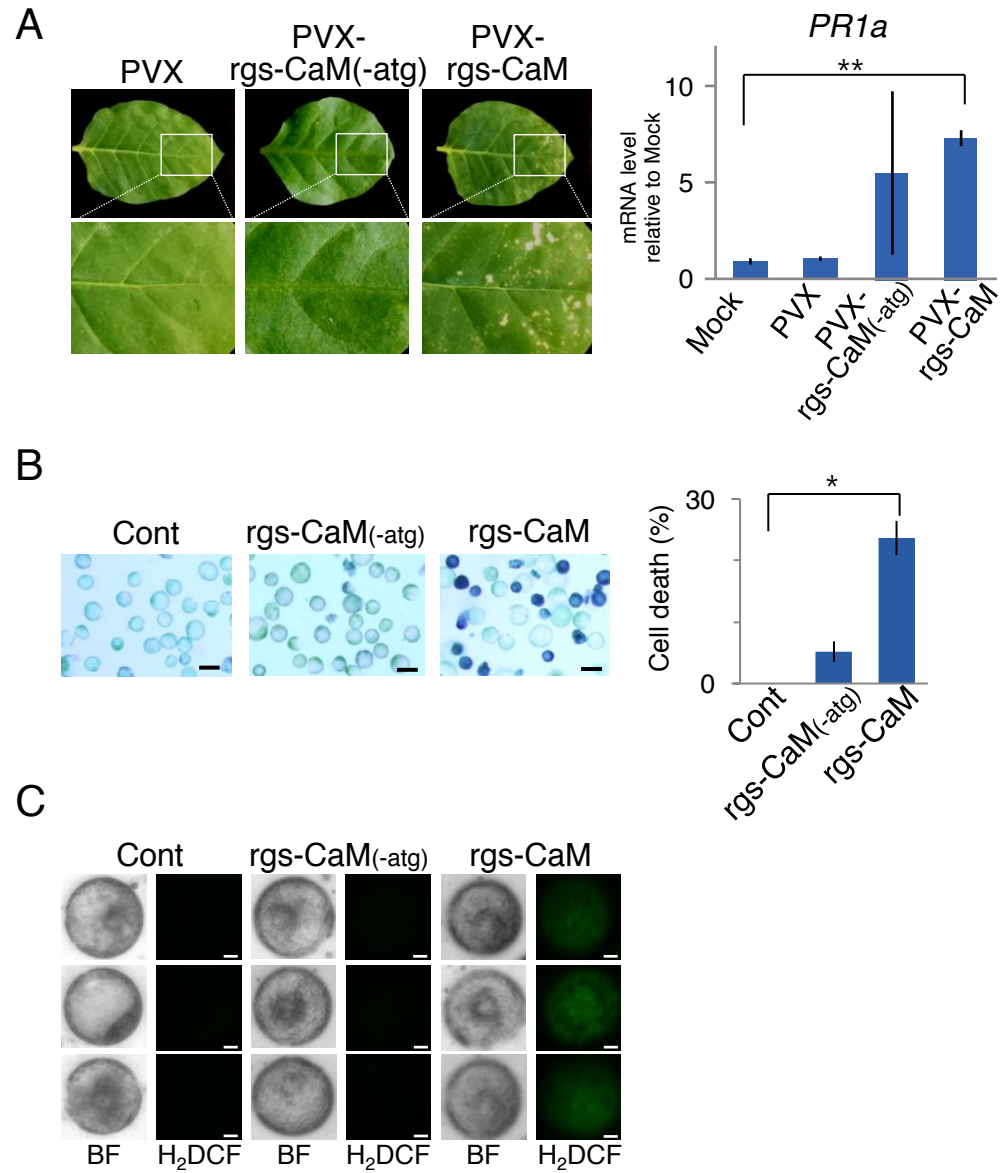


Figure 3

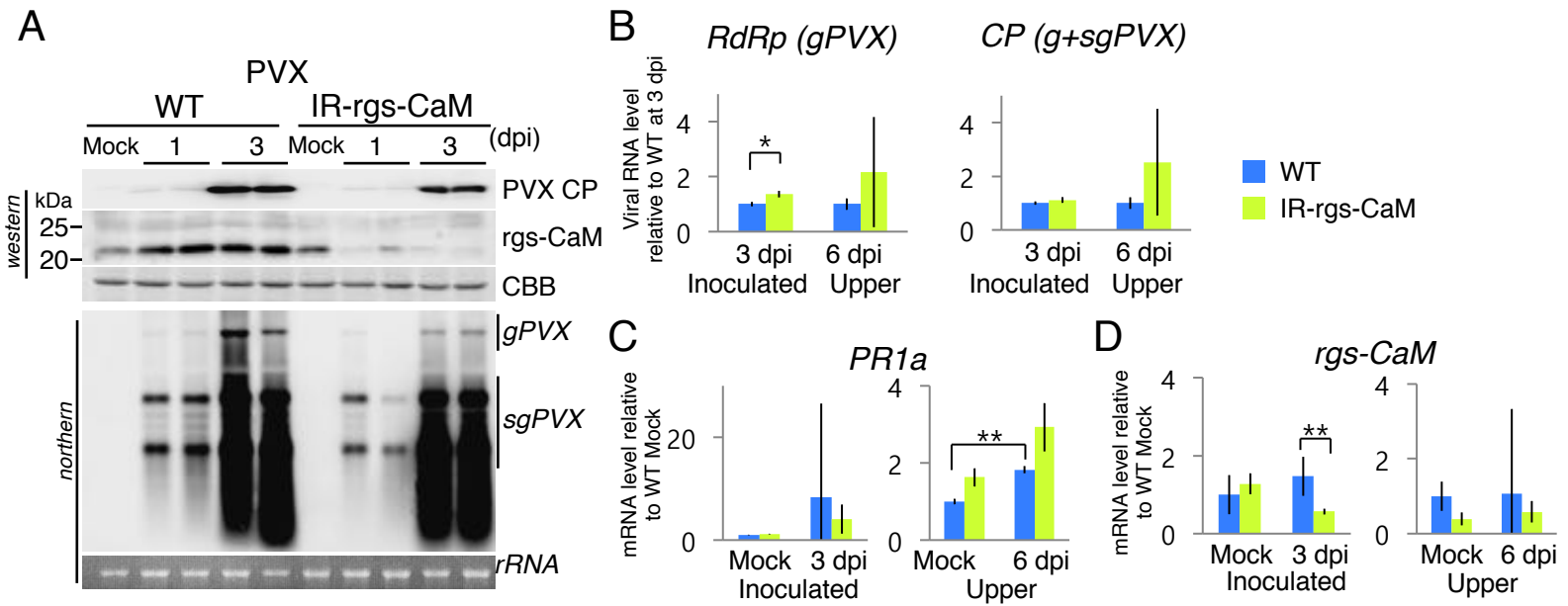
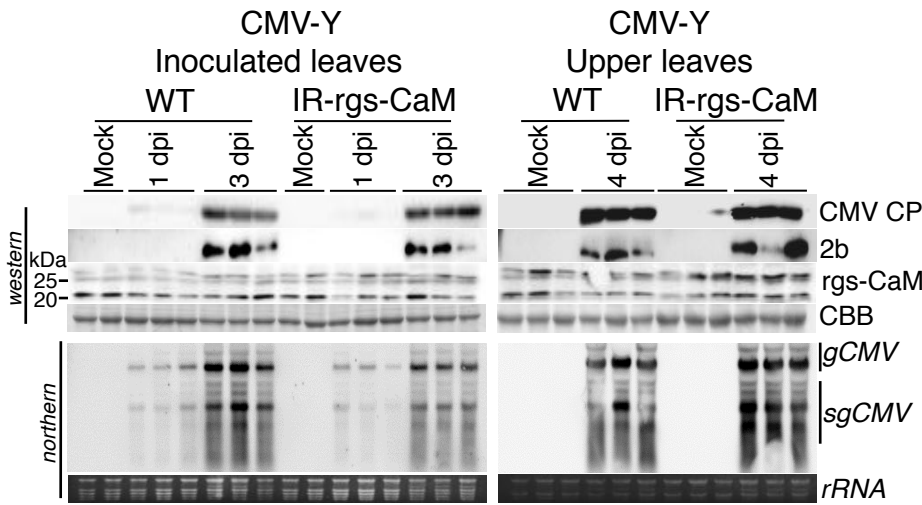
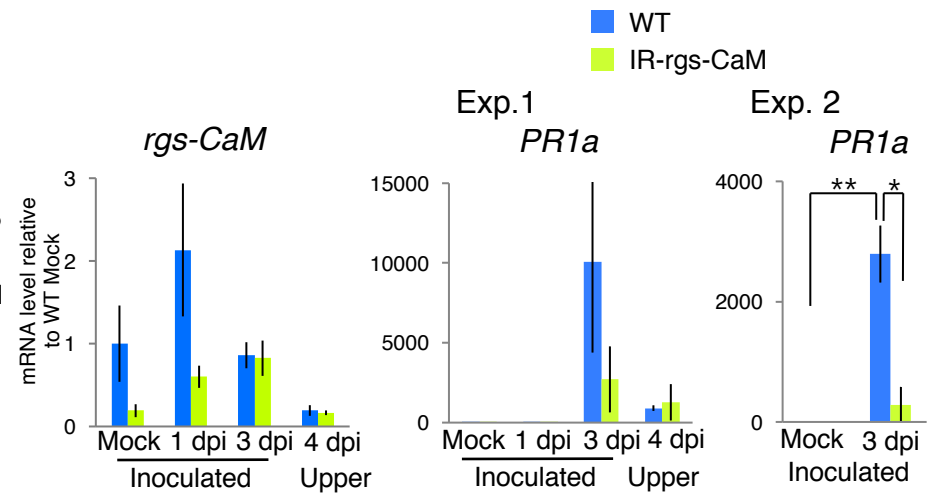


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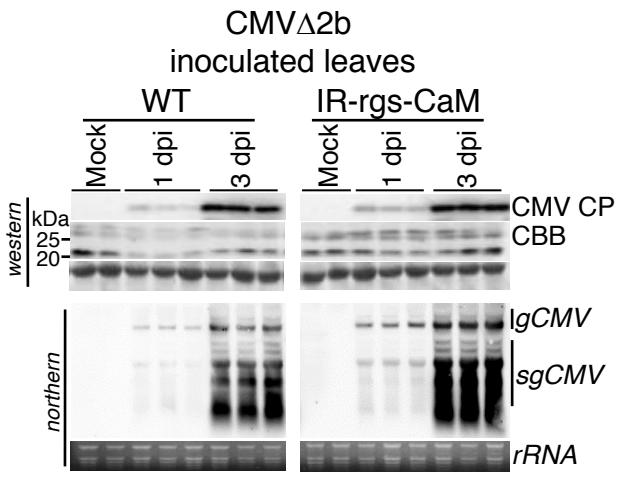
A



B



C



D

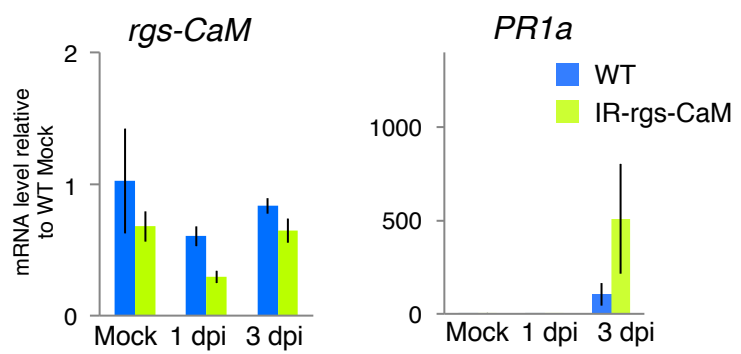


Figure 5

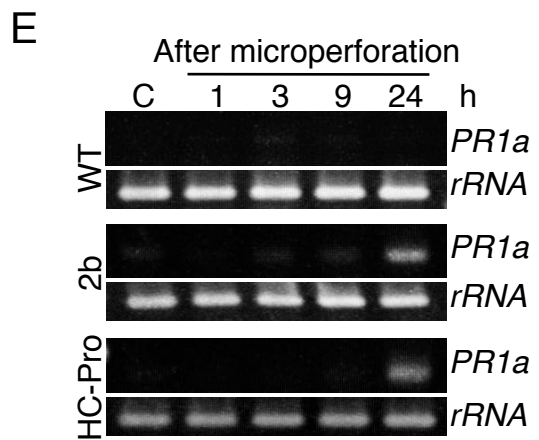
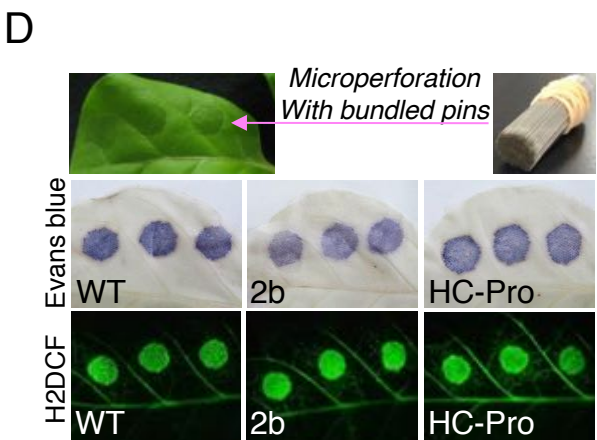
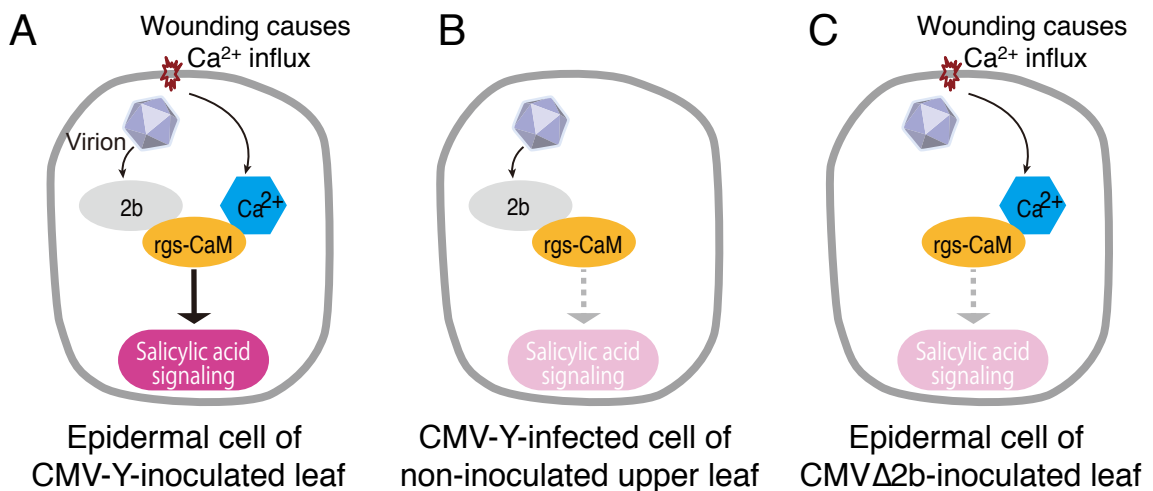




Figure 6

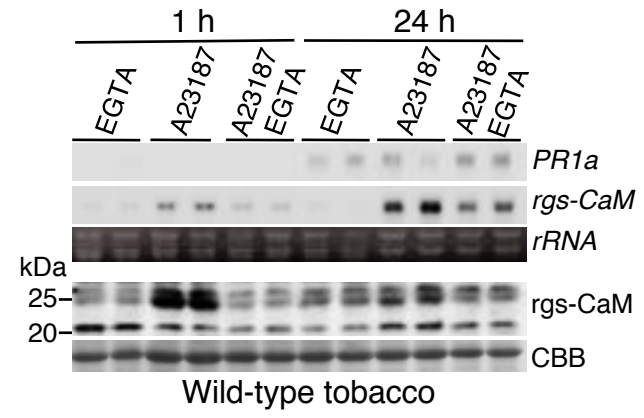
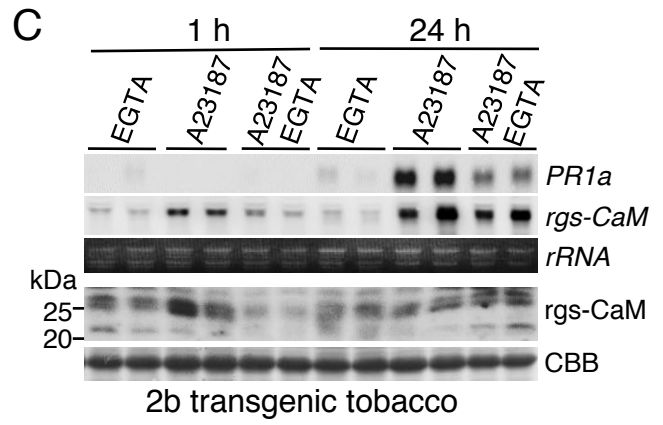
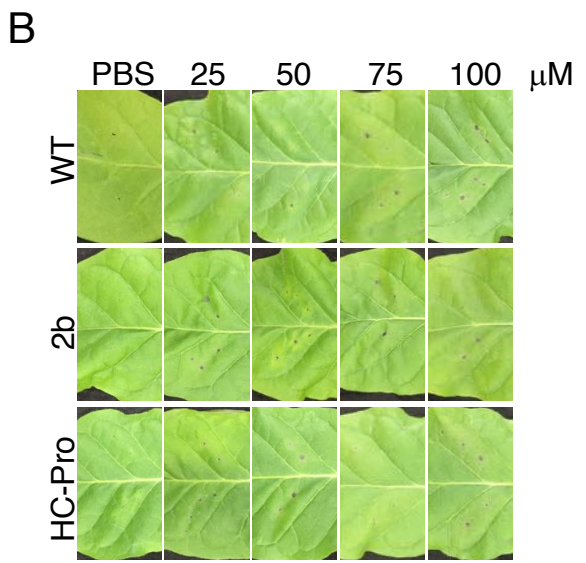
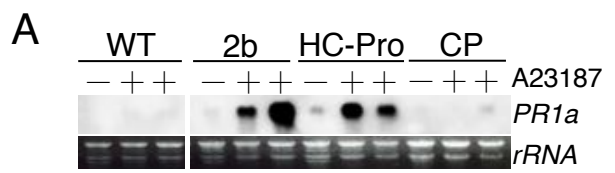


Figure 7

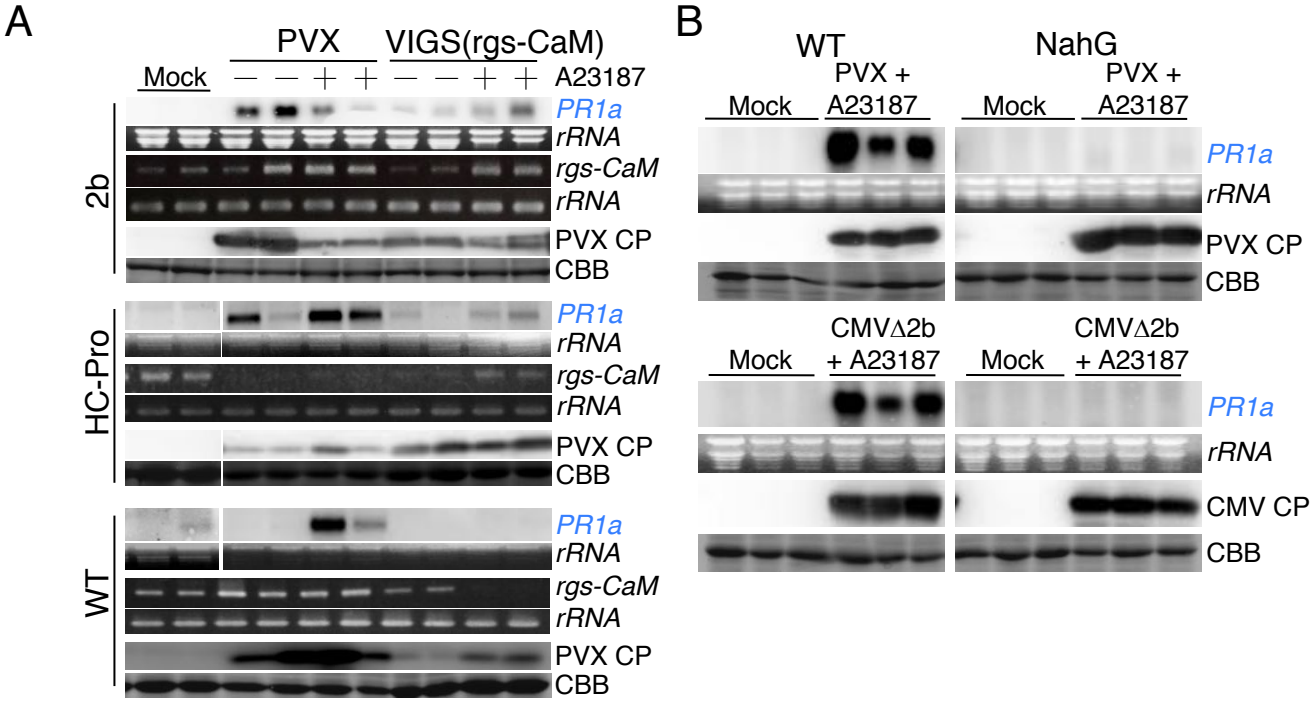
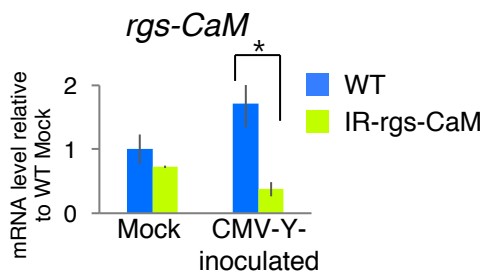
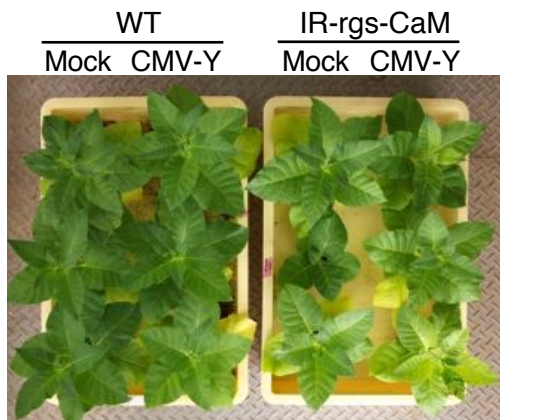
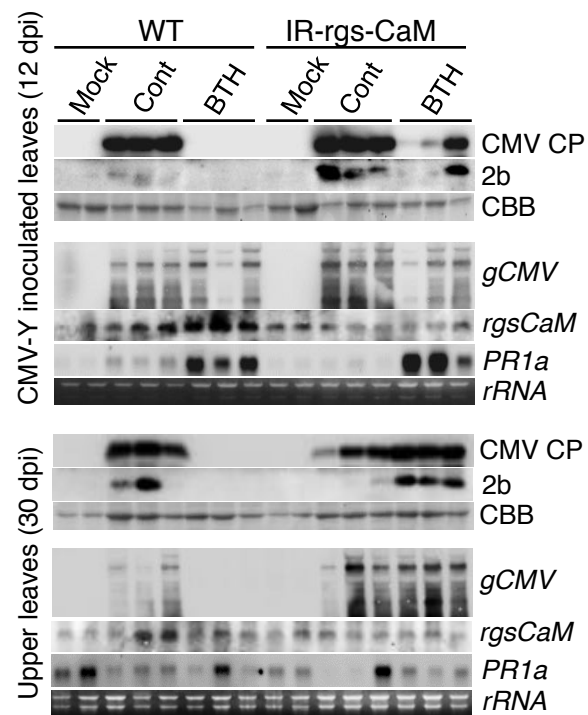
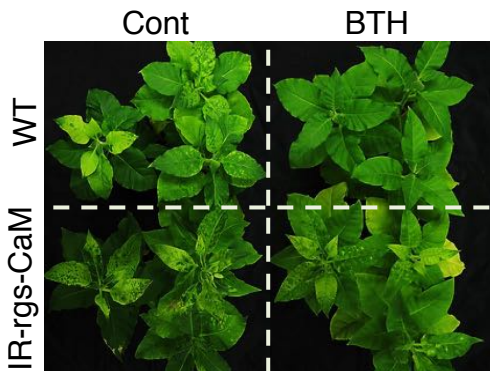


Figure 8

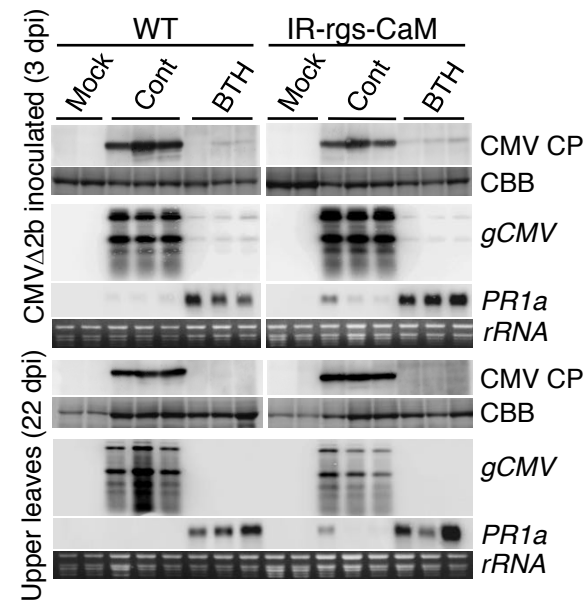
A



B



C



D

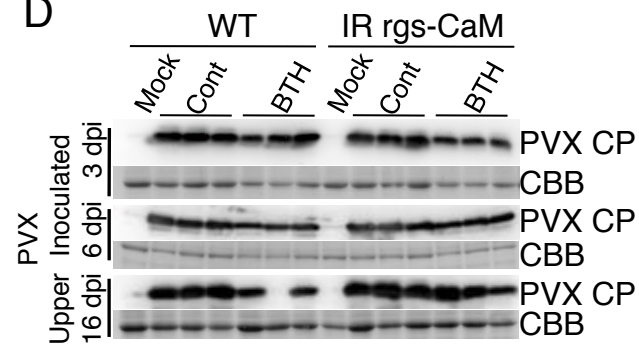
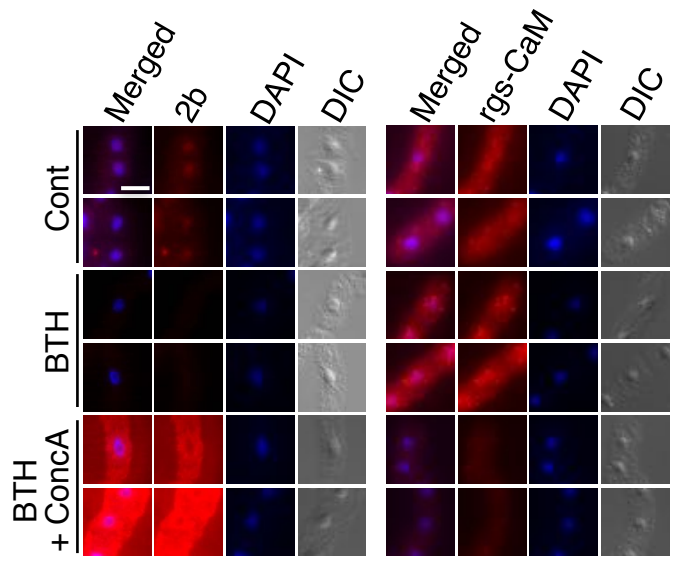


Figure 9

**A**



**B**

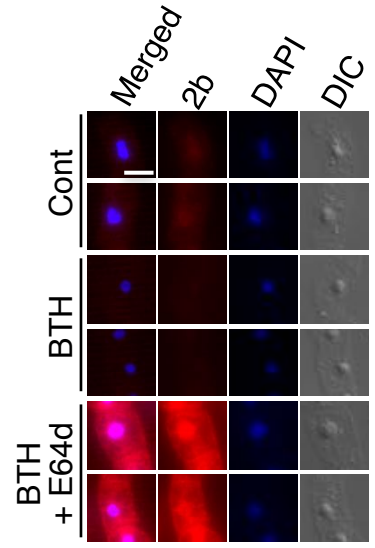
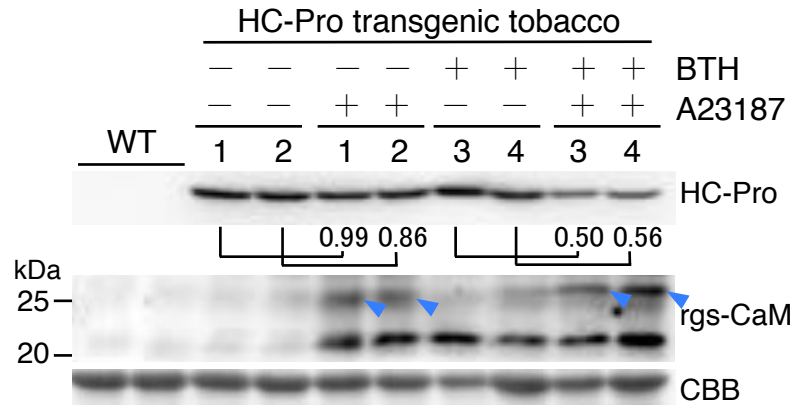
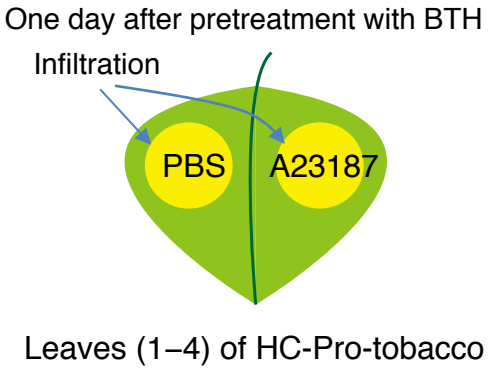
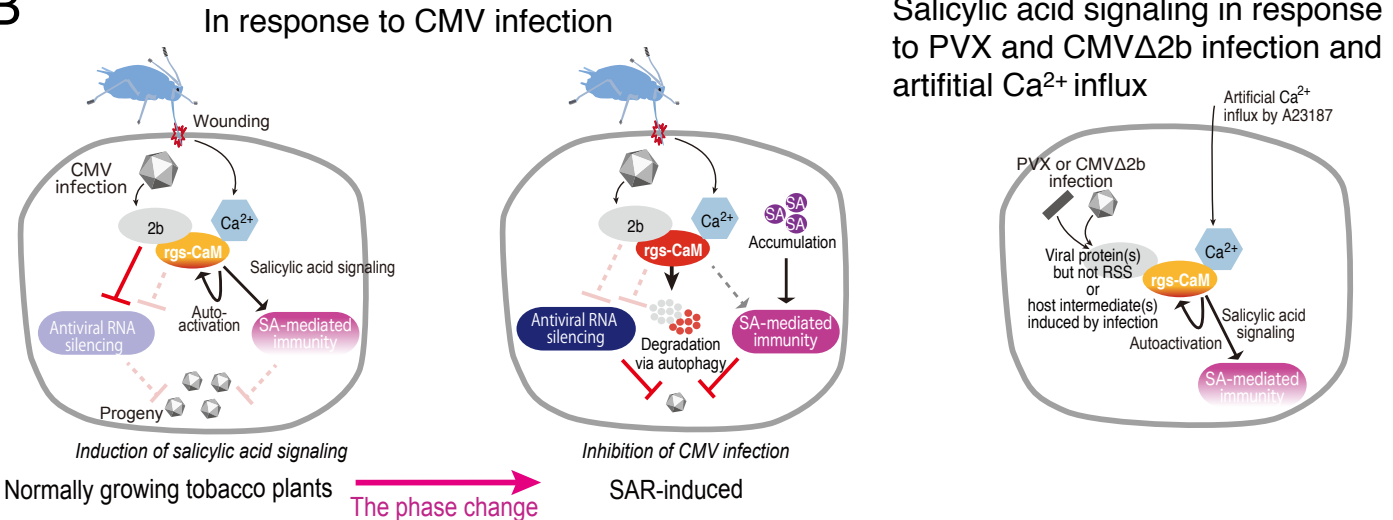


Figure 10

A



B



**Table 1** Primers used for detection of the viral genomic RNAs and endogenous gene expressions

Gene (accession number)	Primer sequences (5'–3')
<i>18S rRNA</i>	F CCGTAGTCCCTCTAAGAAGCTG R GGTCCAGACATAGTAAGGATTG
<i>rgs-CaM</i> (AF329729)	F TGATAGGAGCATTGGAATGTATG R ACTCATCAAAGTTGAGAACTCCATC F ACTATTACTACTGATTATCTTTTCGA (semi-Q-PCR) R CCCAAGGCCAAAGAATTATGTACA (semi-Q-PCR) *F ACTATTACTACTGATTATCTTTTCGA *R GGGATCCTAATACGACTCACTATAGGGGCAAATGCTCCTATCAATTCCT
CaMV 35S promoter	F CCACTGACGTAAGGGATGACGC R GTGTTCTCTCCAAATGAAATGA
<i>PR1a</i> (X06361 Y00707)	F GAAGTGGCGATTCATGACGGCTG R CGAACCGAGTTACGCCAAACCACC *F ATGGGATTTGTTCTCTTTTCACAATTGCC *R AATTCTAATACGACTCACTATAGGGGAAGGTTCTTGATATCAAGCAG
PVX genomic RNA	*F ATGTCAGCACCAGCTAGCACAACA *R AATTCTAATACGACTCACTATAGGGACATTATGGTGGTAGCGTGAC F ACCAATCTTTTACAGACTCCACCAC (for RdRp) R CTCTAGATCATTAGCCGCTTCAACC (for RdRp) F AGGGTCAACTACCTCAACTACCAC (for CP) R TCCTTCCAAATAGCCTCAATCTTGC (for CP)
CMV genomic RNA	*F GGCGGGAGCTGAGTTGGCAGTTCTGC *R AATTCTAATACGACTCACTATAGGGGTCTCCTTTTGGAGGCCCCACGA

\* Primers used for making DIG-cRNA probes for northern blotting

F: Sense primer

R: Antisense primer