



Title	Wound-induced rgs-CaM gets ready for counterresponse to an early stage of viral infection
Author(s)	Tadamura, Kazuki; Nakahara, Kenji S; Masuta, Chikara; Uyeda, Ichiro
Citation	Plant Signaling & Behavior, 7(12), 1548-1551 <a href="https://doi.org/10.4161/psb.22369">https://doi.org/10.4161/psb.22369</a>
Issue Date	2012-12
Doc URL	<a href="http://hdl.handle.net/2115/50867">http://hdl.handle.net/2115/50867</a>
Type	article (author version)
File Information	PSBtadamura2012.pdf



[Instructions for use](#)

1 **Wound-induced rgs-CaM gets ready for counterresponse to an early stage of viral**  
2 **infection**

3  
4 Kazuki Tadamura, Kenji S. Nakahara\*, Chikara Masuta and Ichiro Uyeda

5  
6 Plant Breeding Science, Research Faculty of Agriculture, Hokkaido University,  
7 Sapporo, 060-8589, Japan

8  
9 \*Correspondence to: Kenji S. Nakahara; Email: knakahar@res.agr.hokudai.ac.jp

10  
11 Keywords: plant innate immunity, RNAi, autophagy, calmodulin-like protein, pattern  
12 recognition receptor, PAMPs

13  
14 Plants and animals can recognize the invasion of pathogens through their perception of  
15 pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors  
16 (PRRs). Plant PRRs identified have been exclusively receptor-like kinases/proteins  
17 (RLK/Ps), and no RLK/P that can detect viruses has been identified to date. RNA  
18 silencing (RNA interference, RNAi) is regarded as an antiviral basal immunity because  
19 the majority of plant viruses has RNA as their genomes and encode RNA silencing  
20 suppressor (RSS) proteins to counterattack antiviral RNAi. Many RSSs were reported to  
21 bind to double-stranded RNAs (dsRNAs), which are regarded as viral PAMPs. We have  
22 recently identified a tobacco calmodulin (CaM)-like protein, rgs-CaM, as a PRR that  
23 binds to diverse viral RSSs through its affinity for the dsRNA-binding domains.  
24 Because rgs-CaM seems to target RSSs for autophagic degradation with self-sacrifice,  
25 the expression level of rgs-CaM is important for antiviral activity. Here, we found that

1 the rgs-CaM expression was induced immediately (within 1 h) after wounding at a  
2 wound site on tobacco leaves. Since the invasion of plant viruses is usually associated  
3 with wounding, and several hours are required for viruses to replicate to a detectable  
4 level in invaded cells, the wound-induced expression of rgs-CaM seems to be linked to  
5 its antiviral function, which should be ready before the virus establishes infection.  
6 CaMs and CaM-like proteins usually transduce calcium signals through their binding to  
7 endogenous targets. Therefore, rgs-CaM is a unique CaM-like protein in terms of  
8 binding to exogenous targets and functioning as an antiviral PRR.

### 9

### 10 **Viral PAMPs and Pattern Recognition Receptors of Plants**

### 11

12 Plants employ multiple layers of innate immunity, which result from a coevolutionary  
13 arms race with pathogenic microorganisms.<sup>1</sup> A first layer of the innate immunity  
14 involves the perception of pathogen-associated molecular patterns (PAMPs), which are  
15 usually common among microorganisms including pathogens such as bacterial flagellin,  
16 comprising the flagellum and fungal chitin of its cell wall (PAMPs-triggered immunity,  
17 PTI).<sup>2</sup> PAMPs are not found in host plants. Binding of PAMPs to pattern recognition  
18 receptors (PRRs) usually provokes stereotypical protective reactions, including ion  
19 fluxes, oxidative bursts, the activation of mitogen-activated protein kinases, protein  
20 phosphorylations, several gene activations, and callose deposition.<sup>2</sup> All PRRs identified  
21 so far for bacterial and fungal PAMPs are receptor-like kinases/proteins (RLK/Ps)<sup>3</sup> (**Fig.**  
22 **1**). RLK/Ps are anchored on a plasma membrane of plant cells and monitor  
23 microorganisms in the apoplast. No RLK/P that can detect plant viruses has been  
24 identified to date, perhaps because plant viruses are obligate intracellular parasites; i.e.,  
25 they directly invade plant cells by means of mechanical wounding or through the

1 feeding behavior of viral vector organisms and spread systemically through the  
2 symplast pathway via plasmodesmata, but do not spread through the apoplast pathway.

3 If PTI exists in the interaction between plants and viruses, what are viral PAMPs and  
4 the PAMPs receptors in the cytoplasm of plant cells? RNA silencing (RNA interference,  
5 RNAi) could be regarded as a PAMP-triggered immunity against viruses in plants.  
6 RNAi is a conserved regulation system of endogenous and exogenous RNAs, and their  
7 encoding genes in eukaryotes. RNAi is induced by a double-stranded RNA (dsRNA)  
8 and quenches its cognate RNAs. In plants, RNAi is a general antiviral defense  
9 mechanism.<sup>4</sup> Most plant viruses have been reported to encode RNAi suppressors (RSSs),  
10 which are expressed to facilitate viral infection and multiplication in the invaded plant  
11 cells.<sup>5</sup> Plant RNA viruses form dsRNA in their secondary structures and replicative  
12 intermediates (RIs) in replication; these RNA genomes are thus PAMPs to induce and  
13 targets of RNAi. The RNase-III family ribonuclease Dicers and their interacting  
14 dsRNA-binding proteins have pivotal roles during initial steps of the RNAi, processing  
15 small RNAs from long dsRNAs.<sup>6-8</sup> *Arabidopsis thaliana* has four Dicer-like proteins  
16 (DCL1-4) and five dsRNA-binding proteins (DRB1/HYL1, DRB2-5) that are orthologs  
17 of animal dsRNA-binding proteins that interact with Dicers. Among them, DCL2,  
18 DCL4, and DRB4 have been reported to be involved in antiviral defense.<sup>9,10</sup> DCL4  
19 interacts with DRB4 to generate 21-nt small interfering RNAs (siRNAs) from  
20 exogenous and endogenous long dsRNAs.<sup>11-16</sup> DCL2 generates 22-nt siRNAs from  
21 dsRNAs, but its interacting DRB partner remains to be determined. Since DCL2 and  
22 DCL4 are reported to be hardly capable of binding dsRNAs,<sup>17</sup> recruiting dsRNAs into  
23 the RNAi pathway can be mainly attributed to DRBs. This suggests that DCL2 should  
24 also interact with some DRBs to effectively process dsRNAs. RIs of viral genomes and  
25 the DCL-DRB complexes are thus regarded as viral PAMPs and host PRRs,

1 respectively (**Fig. 1**).

### 3 **Rgs-CaM Binding to Viral RSSs as Viral Secondary PAMPs**

4  
5 We recently identified a tobacco regulator of gene silencing calmodulin-like protein  
6 (rgs-CaM) as another viral PAMPs interactor.<sup>18</sup> The rgs-CaM protein was previously  
7 reported to interact with a RSS protein, HC-Pro, encoded by tobacco etch virus.<sup>19</sup> We  
8 found that rgs-CaM bound not only to the potyviral HC-Pro proteins but also to various  
9 viral RSSs through the affinity to their dsRNA-binding domains. The selection pressure  
10 by antiviral RNAi forced diverse viruses to evolutionarily develop RSSs independently,  
11 and thus these RSSs might be expected to disrupt various RNAi steps/components to  
12 suppress RNAi. However, many RSSs are reported to bind to dsRNAs.<sup>5</sup> Binding to and  
13 sequestering dsRNAs away from the RNAi machinery is thought to be a major strategy  
14 for viral RSSs to suppress RNAi. Therefore, we now consider that viral dsRNA-binding  
15 RSSs and rgs-CaM could serve as a viral secondary PAMP and its PRR, respectively  
16 (**Fig. 1**).

### 18 **Wound-Inducible Expression of rgs-CaM**

19  
20 Our recent work showed that rgs-CaM not only bound to viral RSSs but also attenuated  
21 the anti-RNAi activity of RSSs, presumably by directing degradation of the RSS  
22 proteins via autophagy with self-sacrifice. The more rgs-CaM expressed, the more RSSs  
23 should be degraded. Therefore, this function of rgs-CaM against viral RSSs suggests  
24 that the expression level of rgs-CaM must be important to the degree of resistance  
25 against virus infection. Indeed, transgenic tobacco plants, in which rgs-CaM was

1 overexpressed, showed increased resistance against viruses. Those plants in which  
2 *rgs-CaM* was repressed by RNAi, showed reduced resistance.<sup>18</sup> Therefore, when  
3 *rgs-CaM* effectively functions for defense against viruses, its expression should be  
4 induced immediately after, or in advance of, virus invasion. As noted above, because  
5 plant virus invasion is usually accompanied by wounding, wounding could be one of the  
6 inducers for the *rgs-CaM* expression. Here, we tested whether wounding can induce the  
7 *rgs-CaM* expression. Total RNA was extracted from tobacco leaf tissues 1 and 24 h  
8 after wounding the leaf with a bottle of 200 needles. The mRNA levels of *rgs-CaM* and  
9 a tobacco wound-induced mitogen-activated protein kinase (*WIPK*) were analyzed by a  
10 real-time PCR assay as previously described.<sup>20</sup> The *rgs-CaM* mRNA level was  
11 drastically increased at 1 h after wounding, and also at 24 h, but to a lesser extent (**Fig.**  
12 **2**). Wounding immediately elicits the expression of a number of resistance-related genes  
13 including *WIPK*, which are associated with oxidative and jasmonic acid bursts.<sup>21</sup> The  
14 expression of some genes become maximal within 2-3 h<sup>22</sup> and *rgs-CaM* seems to be one  
15 such early-induced gene. *Arabidopsis* CaM-like proteins (*CMLs*) 37-39, which are the  
16 most similar to *rgs-CaM* among the *CaMs* and *CMLs*, have also been reported to be  
17 wound-inducible.<sup>23</sup> Considering that tobacco mosaic virus (TMV) needs 2-4 h to  
18 establish infection and replicate its progeny to a detectable level in initially infected  
19 cells,<sup>24</sup> and 18-20 h to initiate movement to adjacent cells,<sup>25</sup> wound-induced *rgs-CaM*  
20 seems to be well prepared for defense against viruses in the initial stage of viral  
21 infection.

22

23

### **Rgs-CaM as a PRR for Viruses**

24

25 *CaM* is well conserved among higher organisms and is extensively evolved in plants.

1 While humans have only three *CaM* genes in their genome, *Arabidopsis* has seven  
2 *CaMs* and 50 *CMLs* and rice has five *CaMs* and 32 *CMLs*.<sup>26,27</sup> *CaMs* and *CMLs* were  
3 reported to play crucial roles in plant growth and development, plant-microbe  
4 interactions, plant immunity, and abiotic stress responses.<sup>26</sup> *Rgs-CaM* is the only *CaM*  
5 (*CML*) that binds to an exogenous target that has been identified so far. *CaMs* and  
6 *CMLs* possess EF hand motifs, which bind to calcium ions ( $\text{Ca}^{2+}$ ) to perceive  
7 environmental cues of various biotic and abiotic stresses through  $\text{Ca}^{2+}$  fluxes in the  
8 cytoplasm, and to transduce signals leading to the induction of appropriate responses.<sup>27</sup>  
9 One question is raised: does *rgs-CaM* transduce signals after binding to  $\text{Ca}^{2+}$  and/or  
10 viral RSSs? Binding of *Arabidopsis* *CMLs* 37-39 to  $\text{Ca}^{2+}$  were reported to change the  
11 conformation of *CMLs*.<sup>23</sup> We are now investigating how the  $\text{Ca}^{2+}$  flux is involved in the  
12 antiviral function of *rgs-CaM* if this is the case.

13

14

### Acknowledgments

15 This work was supported by the Japan Society for the Promotion of Science and the  
16 Ministry of Education, Culture, Sports, Science, and Technology of Japan (18108001  
17 and 20688002).

18

19

### References

20

- 21 1. Jones JD, Dangl JL. The plant immune system. *Nature* 2006; 444: 323-9.
- 22 2. Boller T, Felix G. A renaissance of elicitors: perception of microbe-associated  
23 molecular patterns and danger signals by pattern-recognition receptors. *Annu Rev*  
24 *Plant Biol* 2009; 60: 379-406.
- 25 3. Monaghan J, Zipfel C. Plant pattern recognition receptor complexes at the plasma

- 1 membrane. *Curr Opin Plant Biol* 2012; 15: 1-9.
- 2 4. Ding SW, Voinnet O. Antiviral immunity directed by small RNAs. *Cell* 2007; 130:  
3 413-26.
- 4 5. Csorba T, Pantaleo V, Burgyan J. RNA silencing: an antiviral mechanism. *Adv Virus*  
5 *Res* 2009; 75: 35-71.
- 6 6. Lee YS, Nakahara K, Pham JW, Kim K, He Z, Sontheimer EJ, et al. Distinct roles for  
7 *Drosophila* Dicer-1 and Dicer-2 in the siRNA/miRNA silencing pathways. *Cell*  
8 2004; 117: 69-81.
- 9 7. Miyoshi K, Miyoshi T, Hartig JV, Siomi H, Siomi MC. Molecular mechanisms that  
10 funnel RNA precursors into endogenous small-interfering RNA and microRNA  
11 biogenesis pathways in *Drosophila*. *RNA* 2010; 16: 506-15.
- 12 8. Miyoshi K, Miyoshi T, Siomi H. Many ways to generate microRNA-like small  
13 RNAs: non-canonical pathways for microRNA production. *Mol Genet Genomics*  
14 2010; 284: 95-103.
- 15 9. Ding SW. RNA-based antiviral immunity. *Nat Rev Immunol* 2010; 10: 632-44.
- 16 10. Qu F, Ye X, Morris TJ. Arabidopsis DRB4, AGO1, AGO7, and RDR6 participate in  
17 a DCL4-initiated antiviral RNA silencing pathway negatively regulated by DCL1.  
18 *Proc Natl Acad Sci U S A* 2008; 105: 14732-7.
- 19 11. Adenot X, Elmayan T, Laressergues D, Boutet S, Bouche N, Gascioli V, et al.  
20 DRB4-dependent TAS3 trans-acting siRNAs control leaf morphology through  
21 AGO7. *Curr Biol* 2006; 16: 927-32.
- 22 12. Nakazawa Y, Hiraguri A, Moriyama H, Fukuhara T. The dsRNA-binding protein  
23 DRB4 interacts with the Dicer-like protein DCL4 in vivo and functions in the  
24 trans-acting siRNA pathway. *Plant Mol Biol* 2007; 63: 777-85.
- 25 13. Curtin SJ, Watson JM, Smith NA, Eamens AL, Blanchard CL, Waterhouse PM. The

- 1 roles of plant dsRNA-binding proteins in RNAi-like pathways. FEBS Lett 2008;  
2 582: 2753-60.
- 3 14. Dunoyer P, Himber C, Ruiz-Ferrer V, Alioua A, Voinnet O. Intra- and intercellular  
4 RNA interference in *Arabidopsis thaliana* requires components of the microRNA  
5 and heterochromatic silencing pathways. Nat Genet 2007; 39: 848-56.
- 6 15. Jakubiec A, Yang SW, Chua NH. *Arabidopsis* DRB4 protein in antiviral defense  
7 against Turnip yellow mosaic virus infection. Plant J 2012; 69: 14-25.
- 8 16. Fukudome A, Kanaya A, Egami M, Nakazawa Y, Hiraguri A, Moriyama H, et al.  
9 Specific requirement of DRB4, a dsRNA-binding protein, for the in vitro  
10 dsRNA-cleaving activity of *Arabidopsis* Dicer-like 4. RNA 2011; 17: 750-60.
- 11 17. Hiraguri A, Itoh R, Kondo N, Nomura Y, Aizawa D, Murai Y, et al. Specific  
12 interactions between Dicer-like proteins and HYL1/DRB-family dsRNA-binding  
13 proteins in *Arabidopsis thaliana*. Plant Mol Biol 2005; 57: 173-88.
- 14 18. Nakahara KS, Masuta C, Yamada S, Shimura H, Kashihara Y, Wada TS, et al.  
15 Tobacco calmodulin-like protein provides secondary defense by binding to and  
16 directing degradation of virus RNA silencing suppressors. Proc Natl Acad Sci USA  
17 2012; 109: 10113-8.
- 18 19. Anandalakshmi R, Marathe R, Ge X, Herr JM, Jr., Mau C, Mallory A, et al. A  
19 calmodulin-related protein that suppresses posttranscriptional gene silencing in  
20 plants. Science 2000; 290: 142-4.
- 21 20. Atsumi G, Kagaya U, Kitazawa H, Nakahara KS, Uyeda I. Activation of the  
22 salicylic acid signaling pathway enhances Clover yellow vein virus virulence in  
23 susceptible pea cultivars. Mol Plant Microbe Interact 2009; 22: 166-75.
- 24 21. Kessler A, Baldwin IT. Plant responses to insect herbivory: the emerging molecular  
25 analysis. Annu Rev Plant Biol 2002; 53: 299-328.

- 1 22. Reymond P, Weber H, Damond M, Farmer EE. Differential gene expression in  
2 response to mechanical wounding and insect feeding in Arabidopsis. *Plant Cell*  
3 2000; 12: 707-20.
- 4 23. Vanderbeld B, Snedden WA. Developmental and stimulus-induced expression  
5 patterns of Arabidopsis calmodulin-like genes CML37, CML38 and CML39. *Plant*  
6 *Mol Biol* 2007; 64: 683-97.
- 7 24. Watanabe Y, Emori Y, Ooshika I, Meshi T, Ohno T, Okada Y. Synthesis of  
8 TMV-specific RNAs and proteins at the early stage of infection in tobacco  
9 protoplasts: transient expression of the 30K protein and its mRNA. *Virology* 1984;  
10 133: 18-24.
- 11 25. Kawakami S, Watanabe Y, Beachy RN. Tobacco mosaic virus infection spreads cell  
12 to cell as intact replication complexes. *Proc Natl Acad Sci USA* 2004; 101: 6291-6.
- 13 26. Perochon A, Aldon D, Galaud JP, Ranty B. Calmodulin and calmodulin-like  
14 proteins in plant calcium signaling. *Biochimie* 2011; 93: 2048-53.
- 15 27. DeFalco TA, Bender KW, Snedden WA. Breaking the code: Ca<sup>2+</sup> sensors in plant  
16 signalling. *Biochem J* 2010; 425: 27-40.
- 17 28. Moffett P. Mechanisms of recognition in dominant R gene mediated resistance. *Adv*  
18 *Virus Res* 2009; 75: 1-33.
- 19 29. Li HW, Lucy AP, Guo HS, Li WX, Ji LH, Wong SM, et al. Strong host resistance  
20 targeted against a viral suppressor of the plant gene silencing defence mechanism.  
21 *Embo J* 1999; 18: 2683-91.

22

23

24 Figure legends

25

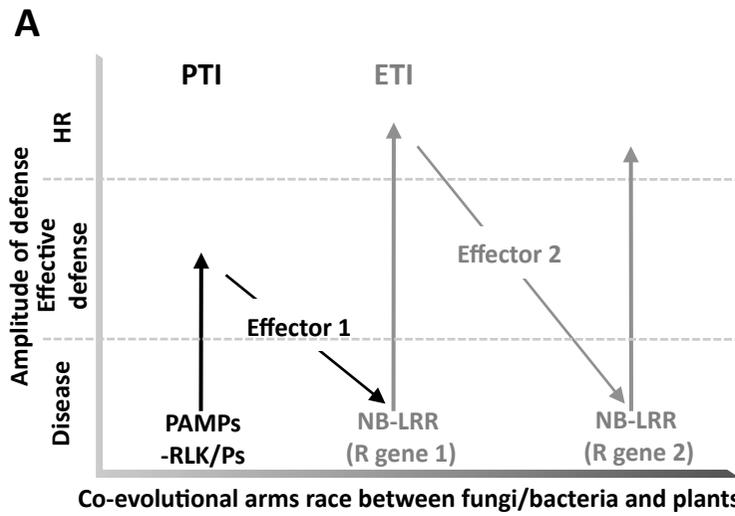
1 **Figure 1.** PAMPs-triggered immunity (PTI) against bacteria and fungi (A) and PTI  
2 against viruses (B) in plants. PTI against bacteria and fungi is illustrated based on the  
3 zigzag model proposed previously.<sup>1</sup> (A) In a first layer of defense, plants recognize  
4 invaded microbes by perceiving pathogen-associated molecular patterns (PAMPs) with  
5 receptor-like kinase/proteins (RLK/Ps) and mount defense reactions (PTI). To colonize  
6 the plants, pathogenic microbes secrete effectors into plant cells to suppress PTI  
7 (effector-triggered immunity, ETI). In a second layer of defense, plants develop  
8 nucleotide binding and leucine-rich repeat proteins (NB-LRRs) to perceive the pathogen  
9 effectors and mount a strong defense, or hypersensitive reaction (HR), which usually  
10 accompanies the generation of reactive oxidative species and programmed cell death.  
11 Pattern recognition receptors (PRRs) for bacterial and fungal PAMPs identified so far  
12 are listed in the table on the right. (B) Based on the PTI against bacteria and fungi, we  
13 here proposed a model of PTI against viruses based on our recent findings regarding the  
14 calmodulin-like protein rgs-CaM.<sup>18</sup> The double-stranded RNA (dsRNA) forms of viral  
15 genomes, which are regarded as viral PAMPs, seem to induce RNA silencing (RNA  
16 interference, RNAi) against viruses. The viral dsRNAs are taken into the RNAi pathway  
17 and processed into small RNAs by Dicer-like proteins (DCL)-dsRNA-binding protein  
18 (DRB) complexes. Most pathogenic viruses counteractively express RNA silencing  
19 suppressor (RSS) proteins to facilitate their infection and multiplication in invaded plant  
20 cells. Many viral RSSs were reported to bind to dsRNA to suppress RNAi.<sup>5</sup> Therefore,  
21 RSSs are considered to be both viral secondary PAMPs, which have dsRNA-binding  
22 domains, and effectors to suppress RNAi (PTI). Rgs-CaM binds to diverse RSSs  
23 through the affinity to their dsRNA-binding domains to sequester RSSs and thus  
24 reinforce RNAi (PTI & ETI). Afterward, plants might develop RSSs that do not bind to  
25 dsRNA and thus rgs-CaM. Some plants are reported to recognize viral RSSs to induce

1 HR.<sup>28,29</sup> The host components considered to be PRRs for viral PAMPs are listed in the  
2 table on the right.

3

4 **Figure 2.** Wound-inducible expression of rgs-CaM. Leaves of wild type tobacco cv.  
5 Bright Yellow were wounded with a bottle of 200 needles. RNAs were extracted from  
6 leaves 1 and 24 h after wounding and those without wounding (control). The mRNA  
7 levels of tobacco calmodulin-like protein (*rgs-CaM*) and wound-induced  
8 mitogen-activated protein kinase (*WIPK*) were investigated using real-time PCR with  
9 the RNA extracts as described previously.<sup>20</sup> Relative expression levels of *rgs-CaM* and  
10 *WIPK* in those leaves with and without wounding were shown in the bar graph. Values  
11 are means  $\pm$ ED of three independent experiments.

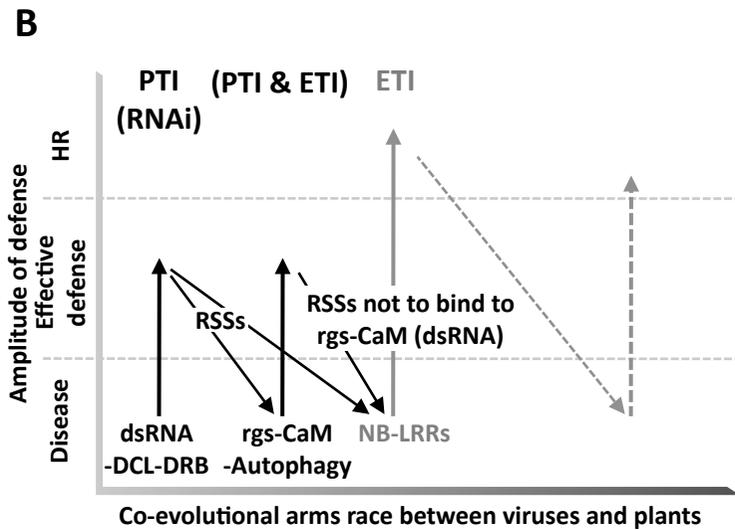
12



Pattern recognition receptors (PRRs) identified in plants

Pathogen types	PAMPs	PRRs	Gene families	Host plants
Bacteria	Flagellin	FLS2	Receptor-like kinase (RLK)	<i>Arabidopsis</i>
	EF-Tu	EFR	RLK	<i>Arabidopsis</i>
	Ax21	XA21	RLK	Rice
Fungi	Peptidoglycan	LYM1	Receptor-like protein (RLP)	<i>Arabidopsis</i>
		LYM3	RLP	<i>Arabidopsis</i>
	Xylanase	Eix1	RLP	Tomato
		Eix2	RLP	Tomato
	Ave1	Ve1	RLP	Tomato
	Chitin	CERK1	RLK	<i>Arabidopsis</i>
		CEBiP	RLP	Rice

PRRs for bacteria and fungi are based on the references.<sup>2,3</sup>



PRRs for viruses in plants

Pathogen types	PAMPs	PRRs	Gene families	Host plants
Viruses	dsRNA	DCL4-DRB4	RNase III and dsRNA binding protein complex	<i>Arabidopsis</i>
		DCL2-DRB?	RNase III and dsRNA binding protein complex	<i>Arabidopsis</i>
	RNA silencing suppressor	rgs-CaM	Calmodulin-like protein	Tobacco

Figure 1

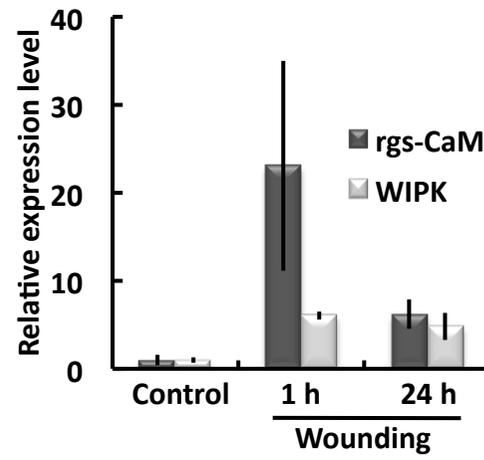


Figure 2