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

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

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

Viral PAMPs and Pattern Recognition Receptors of Plants

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

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

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

respectively (**Fig. 1**).

Rgs-CaM Binding to Viral RSSs as Viral Secondary PAMPs

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

Wound-Inducible Expression of rgs-CaM

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

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

Rgs-CaM as a PRR for Viruses

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

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

Acknowledgments

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References

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

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

roles of plant dsRNA-binding proteins in RNAi-like pathways. FEBS Lett 2008; 582: 2753-60.

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

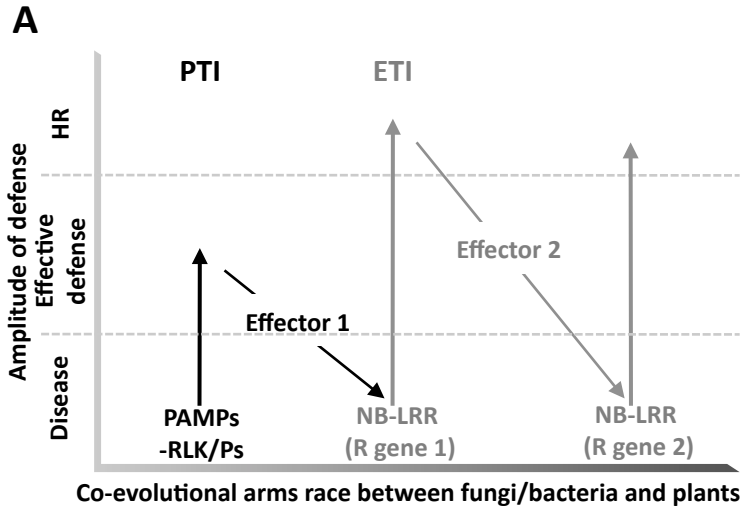
22. Reymond P, Weber H, Damond M, Farmer EE. Differential gene expression in response to mechanical wounding and insect feeding in Arabidopsis. Plant Cell 2000; 12: 707-20.
23. Vanderbeld B, Snedden WA. Developmental and stimulus-induced expression patterns of Arabidopsis calmodulin-like genes CML37, CML38 and CML39. Plant Mol Biol 2007; 64: 683-97.
24. Watanabe Y, Emori Y, Ooshika I, Meshi T, Ohno T, Okada Y. Synthesis of TMV-specific RNAs and proteins at the early stage of infection in tobacco protoplasts: transient expression of the 30K protein and its mRNA. Virology 1984; 133: 18-24.
25. Kawakami S, Watanabe Y, Beachy RN. Tobacco mosaic virus infection spreads cell to cell as intact replication complexes. Proc Natl Acad Sci USA 2004; 101: 6291-6.
26. Perochon A, Aldon D, Galaud JP, Ranty B. Calmodulin and calmodulin-like proteins in plant calcium signaling. Biochimie 2011; 93: 2048-53.
27. DeFalco TA, Bender KW, Snedden WA. Breaking the code: Ca2+ sensors in plant signalling. Biochem J 2010; 425: 27-40.
28. Moffett P. Mechanisms of recognition in dominant R gene mediated resistance. Adv Virus Res 2009; 75: 1-33.
29. Li HW, Lucy AP, Guo HS, Li WX, Ji LH, Wong SM, et al. Strong host resistance targeted against a viral suppressor of the plant gene silencing defence mechanism. Embo J 1999; 18: 2683-91.

Figure legends

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

HR.[28,29] The host components considered to be PRRs for viral PAMPs are listed in the table on the right.

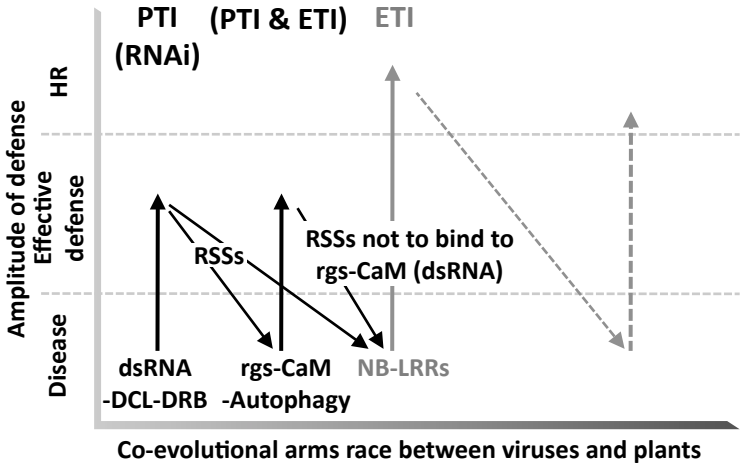
Figure 2. Wound-inducible expression of rgs-CaM. Leaves of wild type tobacco cv. Bright Yellow were wounded with a bottle of 200 needles. RNAs were extracted from leaves 1 and 24 h after wounding and those without wounding (control). The mRNA levels of tobacco calmodulin-like protein (*rgs-CaM*) and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



Pattern recognition receptors (PRRs) identified in plants

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

PRRs for bacteria and fungi are based on the references.[2,3]



PRRs for viruses in plants

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

Figure 1

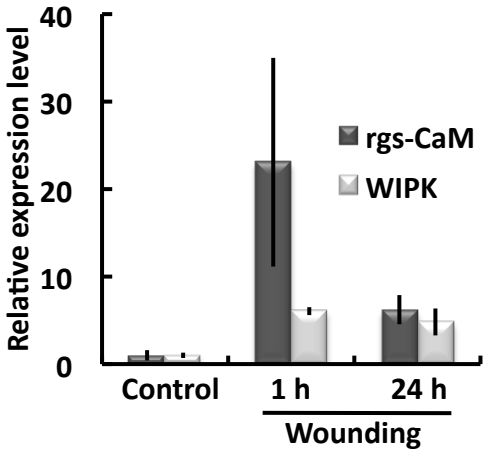

Relative expression level
40
30
20
10
0
rgs-CaM
WIPK
Control
1 h
24 h
Wounding

Figure 2