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. 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). 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. All PRRs identified
so far for bacterial and fungal PAMPs are receptor-like kinases/proteins (RLK/Ps) (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. 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. 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. 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. DCL4
interacts with DRB4 to generate 21-nt small interfering RNAs (siRNAs) from
exogenous and endogenous long dsRNAs. 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, 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.\(^\text{18}\) The rgs-CaM protein was previously reported to interact with a RSS protein, HC-Pro, encoded by tobacco etch virus.\(^\text{19}\) 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.\(^\text{5}\) 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. 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. 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. The expression of some genes become maximal within 2-3 h 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. 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, and 18-20 h to initiate movement to adjacent cells, 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. CaMs and CMLs were reported to play crucial roles in plant growth and development, plant-microbe interactions, plant immunity, and abiotic stress responses. 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. 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. 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

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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.\textsuperscript{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 (\textit{rgs-CaM}) and wound-induced mitogen-activated protein kinase (\textit{WIPK}) were investigated using real-time PCR with the RNA extracts as described previously.\textsuperscript{20} Relative expression levels of \textit{rgs-CaM} and \textit{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

<table>
<thead>
<tr>
<th>Pathogen types</th>
<th>PAMPs</th>
<th>PRRs</th>
<th>Gene families</th>
<th>Host plants</th>
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<tr>
<td>Bacteria</td>
<td>Flagellin</td>
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PRRs for bacteria and fungi are based on the references.²,³

PRRs for viruses in plants

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<th>Gene families</th>
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<td>Calmodulin-like protein</td>
<td>Tobacco</td>
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</tbody>
</table>

Co-evolutional arms race between fungi/bacteria and plants

Co-evolutional arms race between viruses and plants

Figure 1
Figure 2