Linking calcium and RNAi signaling in plants

Yunjing Wang[2,§], Qian Gong[2,§], Zhenhui Jin[1,3,§], Yule Liu[2,*], Yiguo Hong[1,3,4,*]

[1] Research Centre for Plant RNA Signaling, College of Life and Environmental Sciences, Hangzhou Normal University, Hangzhou 311121, China.

[2] MOE Key Laboratory of Bioinformatics, Centre for Plant Biology, Tsinghua-Peking Joint Center for Life Sciences, School of Life Sciences, Tsinghua University, Beijing 100084, China.

[3] School of Science and the Environment, University of Worcester, Worcester WR2 6AJ, UK

[4] School of Life Sciences, University of Warwick, Coventry CV4 7AL, UK

***Correspondence**

yiguo.hong@hzun.edu.cn; yiguo.hong@worc.ac.uk; yiguo.hong@warwick.ac.uk (Hong, Y.)

yuleliu@mail.tsinghua.edu.cn (Liu, Y.)

[§]These authors contributed equally to this work.

The genetic link between calcium signaling and RNA interference (RNAi), has remained undiscovered until now. A new study shows that wound-triggered calcium flux acts as initial messenger for priming RNAi for its role in plant antiviral defense. This paves the way to investigate plant development and response to (a)biotic stresses.

The Question - What is the initial cue to prime RNAi?

RNA interference (RNAi, also known as RNA silencing) is a regulatory mechanism ubiquitous to organisms across kingdoms. Aberrant single-stranded RNA (ssRNA) is converted into double-stranded RNA (dsRNA) by RNA-dependent RNA polymerases (RDRs), such as RDR6 [1]. Subsequently, dsRNA is diced to small interfering RNA (siRNA) of 21, 22 and 24 nucleotides (nt) by DICER in animals, DICER-LIKE endonucleases DCL4, DCL2 and DCL3, respectively in plants or homologous DCLs in fungi [2]. The guide-strand of siRNA together with AGONAUTES (AGOs), for instance AGO1, AGO2 or AGO4, forms RNA-induced silencing complex, which targets specific RNA for cleavage or homologous DNA for RNA-directed DNA methylation [3]. This affects RNA homeostasis and chromatin formation/accessibility which determine gene expression status, resulting in post-transcriptional or transcriptional gene silencing [4]. RNAi can be also triggered by microRNA (miRNA) through miRNA-mediated degradation or translational arrest of mRNA target. In plants, miRNA originate from primary transcripts (pri-miRNAs) with characteristic stem-loop structures through bidirectional processing by DCL1, and this process is remodeled by the ATPase subunit of the large switch/sucrose non-fermentable complex, a partner of the Microprocessor component Serrate [5,6].

The biochemical and genetic framework, including the direct dsRNA trigger, for the intracellular RNAi machinery are well-established [2,7]. Intercellular and systemic RNAi have also been intensively investigated [8]. However, the initial stimuli triggering the cell sensing changes in the environment and subsequently producing dsRNA for RNAi induction remain unknown. Nevertheless, RNAi has profound physiological effects at molecular, cellular, tissue, organ, and organism levels. In plants, RNAi participates in almost all biological/physiological processes and plays essential roles in anti-pathogenic defense, cellular response to environmental changes, transition from vegetative to reproductive growth, modulation of flowering time and development of root, flower, fruit, and seed [4,9,10]. Therefore, to understand both how plants perceive early stimulus for RNAi in absence of the immediate dsRNA trigger and how plants cascade the initial signal to second messenger at the onset of silencing are essential to fully appreciate the broad significance of RNAi in plant physiology. Such insights into plant signal transduction may shed light on RNAi machinery in animals and other organisms such as fungi. Thus, the burning question is what the early stimulus is for signal transduction in RNAi.

The Answer - Bridging the link between intracellular Ca^{2+} signaling and RNAi

Signal transduction is a process by which a chemical or physical signal is transmitted through a cell as a series of molecular events [11]. A potential association of calcium (Ca^{2+}) signal with RNAi may be possible. An early study on repressor of gene silencing (rgs) reveals that a calmodulin (CaM)-like (CML) protein rgsCaM can inhibit antiviral RNAi in plants, inferring the possible involvement of CaM signaling in the RNAi process [12]. However, plants such as

Arabidopsis thaliana encodes numerous CMLs, some of which are not relevant to Ca^{2+} signal transduction. Thus, there has not been clear evidence to show the direct association between Ca^{2+} signal transduction and RNAi, and it remains unknown if Ca^{2+} signaling is indeed involved in RNAi.

A recent study has uncovered that Ca^{2+} can act as a very first (and direct) messenger in signal transduction for RNAi in plants [13]. Plant cells can sense extracellular physical cues, i.e., abiotic wound or insect injury to cells caused during the very early stage of RNA and DNA virus infection, to trigger a rapid elevation in cytosolic Ca^{2+} fluxes, which in turn induce expression of *CaM3* and *CaM-binding transcriptional activator3* (*CAMTA3*), two core components in decoding Ca^{2+} signal (Figure 1). CaMs including CaM3 are one of three major types of Ca^{2+} sensors or Ca^{2+}-binding proteins (CaM/CMLs, Ca^{2+}-dependent protein kinases, and calcineurin B-like proteins) in plants. These Ca^{2+}-binding proteins together with their regulated target proteins such as CAMTA3 are involved in Ca^{2+} signaling, which facilitates plant adaptation to changing environments [14]. After elevation by the wound-induced Ca^{2+} fluxes, CaM3 is found to interact with CAMTA3 in a Ca^{2+}-dependent manner. Such interaction leads to activation of the CAMTA3 functionality and makes CAMTA3 biologically active. CaM3-activated CAMTA3 binds directly to the CGCG box in the promoters of *RDR6* and *Bifunctional nuclease2* (*BN2*), two essential genes in RNAi pathway, and stimulates their transcription. The elevated *RDR6* and *BN2* enhance intracellular RNAi in plants (Figure 1). Here two different pathways, although not mutually exclusive, may cause the intracellular RNAi enhancement. First, up-expressed RDR6 converts ssRNA into more dsRNA, the immediate trigger for RNAi. Second, BN2 prompts degradation of miR162, miR168 and miR403 that target *DCL1*, *AGO1* and *AGO2* mRNAs, respectively, leading to maintenance and even increase in DCL1, AGO1 and AGO2, which are three core components in miRNA-mediated RNA silencing (Figure 1). These findings firmly link Ca^{2+} signaling to intracellular RNAi within the initially wounded cells or the first cells that have sensed the initial abiotic or biotic stimuli in plants. Moreover, Ca^{2+} along with mobile siRNAs signals generated in the initially wounded cell can travel to its neighbouring cells to trigger intercellular and systemic Ca^{2+}—RNAi signaling (Figure 1).

The Function: Involvement of Ca^{2+}—RNAi signaling in antiviral defense

The current study has profound implications in plant-pathogen arms-race in terms of plant RNAi-based defense against viruses [13]. After detecting abiotic wound, plant cells respond with a robust cytosol Ca^{2+} signal to activate multiple antiviral RNAi genes and prime to be ready for combating virus invasion (Figure 1). Indeed, plants with knockdown or knockout of either the core Ca^{2+} signaling genes *CaM3* or *CAMTA3*, or the key RNAi-regulator genes *RDR6* and *BN2* become more susceptible to multiple DNA and RNA virus infection. The involvement of Ca^{2+} signal transduction in antiviral RNAi is further evidenced by the fact that different viral suppressors of RNA silencing (VSR), the geminivirus V2 proteins can disrupt CaM-CAMTA3 interaction. Consequently, CAMTA3-mediated transcriptional activation of both *RDR6* and *BN2* is impaired. Thus, viruses have evolved a specific strategy to counteract the plant defense priming. By analogy to the very early stage of viral infection, this work also reveals that wounding may act as the initial cue for cells to elicit RNAi through Ca^{2+}—CaMs—CAMTA3—BN2/RDR6 signaling cascade to defend against viruses except these transmitted by seeds in plants (Figure 1).

The Prospect: Is Ca^{2+}—RNAi signaling of broad relevance to plant physiology?

Ca^{2+} signal transduction and RNAi are involved in a wide range of plant physiology and both impose extensive impacts on innate defense against various pathogens and pests, cellular response to environmental stresses, and plant growth and development [4,9,10,14,15]. Indeed, abiotic environmental cues and biotic stresses can trigger rapid change of cellular concentration of Ca^{2+} and subsequent signaling response, which can prime the regulatory RNAi machinery in plants. These factors include sunlight, temperature, wind, water (rain) deficiency, wound, and infection by pathogens including viruses, bacteria, fungi, and nematodes, as well as infestation pests such as invertebrate insects. This implies that Ca^{2+}—RNAi signaling is of a broad relevance to plant physiology. Thus, the current work prompts several new research frontiers on the role of Ca^{2+}—RNAi signaling in (i) plant response to water shortage/drought, extreme temperatures, wind and photoperiod/circadian changes, (ii) plant defense against non-viral pathogens and pests, and (iii) phytohormone metabolism and gene expression that affect organogenesis, vegetative vs reproductive transition, flower, fruit and seed development, growth, yield and senescence under changing climates and environments (Figure 2). Due to its profound and broad impact on plant physiology, any feedback control in Ca^{2+}—RNAi signal transduction to balance such essential signaling pathway is also worth further investigations.

Acknowledgments

This work was in part supported by funding from the Ministry of Science & Technology of China (Key International R&D Program 2017YFE0110900 to Y.H.), the National Transgenic Program of China (2016ZX08009001-004 to Y.H.) and National Natural Science Foundation of China (32130086 and 31920103013 to Y.L. and 31872636 to Y.H.).

Declaration of interests

No interests are declared.

Author Contributions

All authors were involved in discussing the overall structure of this Forum, drafting, writing and revising the article. Y.W., Q.G. and Z.J. wrote up The Answer and The Function; as well as contributed to draw Figures. Y.L. and Y.H. formularized The Question, conceptualized The Prospect, and finalized the entire manuscript.

References

1. Dalmay, T. *et al.* (2000) An RNA-dependent RNA polymerase gene in Arabidopsis is required for posttranscriptional gene silencing mediated by a transgene but not by a virus. *Cell* 101, 543–553
2. Henderson, I.R. *et al*. (2006) Dissecting Arabidopsis thaliana DICER function in small RNA processing, gene silencing and DNA methylation patterning. *Nat. Genet.* 38, 721–725
3. Fang, X. and Qi, Y. (2016) RNAi in plants: An Argonaute-centered view. *Plant Cell* 28, 272–285
4. Baulcombe, D.C. (2019) How virus resistance provided a mechanistic foundation for RNA silencing. *Plant Cell* 31, 1395–1396
5. Wang, Z. *et al.* (2018) SWI2/SNF2 ATPase CHR2 remodels pri-miRNAs via Serrate to impede miRNA production. *Nature* **557,** 516–521.
6. Zhu, H. et al. (2013) Bidirectional processing of pri-miRNAs with branched terminal loops by Arabidopsis Dicer-like1. *Nat. Struct. Mo.l Biol.* 20, 1106-1115

7. Tang, G. *et al.* (2003) A biochemical framework for RNA silencing in plants. *Genes Dev.* 17, 49–63

8. Zhang, X. et al. (2019) Revisiting mobile RNA silencing in plants. *Plant Sci.* 278, 113-117

9. Wei, L. et al. (2014) Dicer-like 3 produces transposable element-associated 24-nt siRNAs that control agricultural traits in rice. *Proc. Natl. Acad. Sci. USA* 111, 3877–3882

10. Hong, Y. and Jackson, S. (2015) Floral induction and flower formation--the role and potential applications of miRNAs. *Plant Biotechnol. J.* 13, 282-292

11. Bradshaw, R.A. and Dennis, E. A. (2010) Handbook of Cell Signaling (2nd ed.). eds. Amsterdam, Netherlands: Academic Press

12. Anandalakshmi, R. et al. (2000) A calmodulin-related protein that suppresses posttranscriptional gene silencing in plants. *Science* 290, 142–144

13. Wang, Y. et al. (2021) A calmodulin-binding transcription factor links calcium signaling to antiviral RNAi defense in plants. *Cell Host & Microbe* 29, 1393-1406.e7

14. Kudla, J. et al. (2010) Calcium signals: the lead currency of plant information processing. *Plant Cell* 22, 541-563

15. Braam, J. and Davis, R.W. (1990) Rain-, wind- and touch-induced expression of calmodulin and calmodulin-related genes in Arabidopsis. *Cell* 60, 357-364

Figure Legends

Figure 1. Ca^{2+}—RNAi signal transduction pathway in antiviral defense. Wounding or virus invasion triggers primary (1st) and subsequently causes production of cellular secondary (2nd) Ca^{2+} fluxes. This leads to induce expression of CaMs which physically interact with and activate CAMAT3, a transcription factor. Activated CAMTA3 can then bind to the *BN2* and *RDR6* promoters (PRO^{BN2} and PRO^{RDR6}, respectively) to turn on *BN2* and *RDR6* transcription. BN2 works on microRNAs that target either DCL1 or AGO1/2. Subsequently intracellular post-transcriptional gene silencing (RNAi) is primed by these core RNAi genes. Such intracellular RNAi can spread from cell-to-cell via plasmodesmata (PD) and systemically over long distance through sieve element to trigger intercellular and systemic RNAi through mobile Ca^{2+} (dot) and/or siRNA (=) signals. RNAi can be suppressed by viral suppressors of RNA silencing (VSR)-mediated blockage of Ca^{2+}—RNAi signaling [13] or CaM-like (CML) [12]. Solid-line arrow and cross/T-sign indicate positive or negative impact, respectively. Dash-line with diamond or arrow end or question mark indicates potential involvement in these processes. Colours and thickness of various arrow and/or diamond lines bear no biological implication. This figure was created using BioRender (https://biorender.com/).

Figure 2. Ca^{2+}—RNAi signaling in plants. A simplified model and its biological relevance are proposed. Arrow or the T-sign indicates positive or negative impact on the described events. Question mark shows potential effect of Ca^{2+} signals on CaM-like (CML) expression and function, forming a possible feedback control in Ca^{2+}—RNAi signaling in plants.