Letter to the Editor

Extracellular RNA biology in plants: controversial or just unexplored?

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We submit this letter in response to the editorial commentary by Sabrine Nasfi and Karl-Heinz Kogel (1) on our research article titled "*Arabidopsis apoplastic fluid contains sRNA- and circular RNA-protein complexes that are located outside extracellular vesicles*" recently published in *The Plant Cell* (2).

First, we thank Nasfi and Kogel for highlighting the pressing need to develop sustainable solutions for crop protection using RNA-based technologies. We also thank Nasfi and Kogel for providing a comprehensive overview of our article and how our results contribute to the current knowledge of extracellular RNAs (exRNAs) in plants, and how our conclusions differ from previous studies (3).

Although exRNAs have been extensively researched in mammalian systems, mechanisms of RNA secretion are still poorly understood, with many studies leading to contradictory conclusions. Multiple studies from both mammalian and plant systems have conclusively reported that sRNAs are primarily secreted inside extracellular vesicles (EVs) (3,4), which enable secretion either by direct blebbing from the plasma membrane or by fusion of multivesicular bodies with the plasma membrane. Such a mechanism is attractive because vesicles would protect RNAs from extracellular RNases and might facilitate uptake by target cells, or even other organisms. However, our recent findings suggest that most exRNAs are located outside EVs, and only a small proportion is intravesicular (2). Moreover, to be stable in the extracellular space, this RNA must be protected against degradation by extracellular RNases, either through association with RNA-binding proteins (RPBs), or due to the formation of secondary structures (in the case of circular RNAs and tRNAs) (2). This finding is consistent with recent results from mammalian systems, confirming that most exRNAs are derived from diverse sources and are located outside vesicles (5). This is an important observation, as it strongly suggests that intercellular transport and delivery of RNAs may not require EVs.

Many fungal species have been shown to take up naked RNA from the environment, likely through endocytosis (6). We speculate that this may simply be a way for fungi to acquire nutrients (phosphate and reduced nitrogen), which may result in the uptake of long and sRNAs in a non-sequence-specific manner. Once taken up, however, if the RNA can avoid degradation, it can potentially engage host cell regulatory pathways (e.g., the RNA interference machinery in eukaryotic cells and possibly interfering with translation in bacterial cells). Exchange of stable exRNAs may have thus evolved into an inter-organism communication system. This would add another layer to plant immunity, positioning exRNAs as an early signal in plant-microbe interactions, potentially protecting plants against pathogens and shaping the microbiome. Comprehensive studies focused on identifying targets of naturally secreted exRNAs in different interacting organisms (pathogens and commensals) should provide insight into the regulatory roles of these molecules in inter-organism interaction, if any. Moreover, based on the study conducted by Keller *et al.* (7), EVs might also play a critical role independent of RNA through acting as decoys and taking up toxins or effectors released by pathogens, forming a cellular barrier; however, this potential role needs further

investigation. It is worth noting that pathogens have evolved strategies to suppress RNA silencing pathways in their hosts, which suggests that generation of silencing RNAs in the host plays an important role in immunity (8).

As noted in the commentary by Nasfi and Kogel, our conclusion that most sRNAs are located outside vesicles (2,7) differs from previously published work (3). We attribute this disparity to the differences in what materials were analyzed. In our work, we used sRNAseq and denaturing polyacrylamide gel electrophoresis to compare the sRNA content in the supernatant versus the pellet after ultracentrifugation of extracellular wash fluids at 40,000 g, while previously published work did not analyze the supernatant. We found that all known plant EV proteins markers were present in the 40,000 g pellet and absent from the supernatant, while the majority of sRNAs were left in the supernatant, indicating that most sRNAs are not associated with EVs. By using reverse-transcriptase PCR, however, it is possible to detect specific sRNAs in the pellet as well. Thus, we do not claim that no extracellular sRNAs can be found in EVs, just that the majority are not. But, if the majority are not associated with EVs, it argues that most sRNAs are secreted by an EV-independent mechanism and suggests that these EV sRNAs are more likely to impact gene regulation in other organisms, simply due to their greater abundance.

A second difference between our work and previously published work that was noted in the commentary is that in our work, we analyzed exRNAs isolated from non-infected plants only, while previous work looked at exRNAs isolated from fungal-infected plants. We concur that exRNA content may change in response to infection, and especially in response to host cell death. However, our data suggest that exRNA is relatively stable, and thus unlikely to change rapidly in response to infection. To test this assumption, we are currently assessing how the entire exRNAome and exProteome changes in response to various pathogen elicitors as well as in response to the phytopathogens at different stages of infection. We believe that this combinatorial approach will allow us to establish a better correlation between the secretion of RBPs and a diverse range of exRNAs in response to biotic stress and address some of the previously unanswered questions. Furthermore, screening of fungal mutants deficient in various uptake and RNA processing pathways may allow us to identify the mechanisms involved in transkingdom RNA interference on the pathogen side, which are poorly understood.

Although most exRNA appears to be located outside of EVs, what is noteworthy is that it is not fully digested by RNases, with the majority of exRNA being longer than 70 nucleotides. The mere presence of this RNA in the extracellular space raises several fundamental questions regarding the mechanisms of secretion, stabilization, transport, uptake, and function, most of which also apply to human exRNA. It is an exciting time in exRNA research, especially when one looks 'outside the bubble' (EV).

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Footnote

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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