Conclusive Evidence of Replication of a Plant Virus in Honeybees Is Lacking

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The recent article by Li et al. (1) lacks adequate evidence to support the authors' assertion that a plant virus propagates or replicates in honeybees. Instead, it is possible that tobacco ringspot virus (TRSV) virions associate with the honeybee and parasitic *Varroa* mites in the absence of TRSV replication.

First, the presence of plant viral sequences in an organism does not imply replication. For example, plant viruses have been reported to abound in the human large intestine (2, 3). The genome sequences and the reverse transcription (RT)-PCR products reported by Li et al. could have been generated from TRSV virions on the surface of, or inside, the honeybee or on pollen. As TRSV is pollen transmitted (4, 5), association of TRSV with honeybees via pollen is a distinct possibility.

The only experiment the authors presented to test directly for virus replication was detection of negative-strand TRSV RNA, which indeed should be present only if RNA replication occurs. However, a crucial control was missing, and thus the results do not rule out the possibility that the intended negative-strand-specific RT-PCR actually amplified positive-strand RNA template. Because the authors did not use positive-strand RNA as a negative-control template, amplification of the positive strand by low levels of mispriming on the positive strand cannot be ruled out. The authors used tagged primers to reduce this possibility; however, this technique requires extensive preliminary validation, which was not provided. Even when tagged primers are used, negative-strand specificity can be lost in the presence of high concentrations of positive-strand RNA (6). A further complication is that even if the primers are negative strand specific, it is possible that minute, but amplifiable, quantities of negative-strand RNA are encapsidated in virions (6). The use of RNA from purified TRSV virions as the template would have addressed this possibility.

No *in situ* hybridization was performed to visualize TRSV inside honeybee cells, and no negative-control assays were performed on *in situ* hybridization in *Varroa* mites to verify the specificity of the probe. Importantly, the authors did not show an increase in TRSV levels over time, for example, by inoculating TRSV-free honeybees with TRSV to test infectivity.

What else, besides virus replication, might explain the presence of TRSV in multiple bee tissues? It is feasible that these observations could result from contamination from the gut or cuticle of the bee by virions or TRSV-containing pollen. These possibilities may seem remote, but one could argue the same about the authors' claim that a positive-sense RNA plant virus could replicate in insects, which is something that has not been reported previously, despite decades of research on plant virus-insect interactions. Rather than debate which explanation is more likely, controlled experiments that conclusively reveal or rule out replication would answer the question. In summary, we are not stating that TRSV does not replicate in honeybees, but we conclude that the evidence presented by Li et al. to support their claim is inconclusive. For another analysis, see episode 271 of *This Week in Virology* (7).

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