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NemaTox: Targeting root-knot nematodes through plastid-based Bt delivery

Plant parasitic nematodes are microscopic round worms that cause substantial damage to global agriculture, leading to an estimated $150 billion in annual losses (Nicol et al., 2011). Among these nematodes, root-knot nematodes (RKNs) (*Meloidogyne* spp.) are particularly destructive due to their broad host range, affecting over 2000 plant species worldwide. RKNs establish specialized feeding structures called giant cells within the vasculature of host roots. Once these giant cells are formed, the nematodes become sedentary for the rest of their life cycle. Giant cells provide nematodes with essential nutrients required for their growth and development. These cells are enriched with organelles such as mitochondria, endoplasmic reticulum, and plastids, supporting the nematode's metabolic needs. The formation of giant cells disrupts normal root function, leading to the characteristic tumor-like galls associated with RKN infection (Lin and Siddique, 2024).

Current pest management strategies—such as crop rotation, resistant cultivars, and pesticide application—often show limited success against plant-parasitic nematodes due to their broad host range and the scarcity of resistant crop varieties. Moreover, increasing environmental concerns have led to the phasing out of effective chemical nematicides, leaving growers with fewer options to control these pests. This underscores the need for sustainable, environmentally friendly alternatives to manage nematode infestations effectively (Desaeger et al., 2020). *Bacillus thuringiensis* (Bt) is a bacterium that produces a toxin lethal to various insect pests such as European and southwestern corn borers (Peferoen, 1997; Tabashnik and Carrière, 2017). Bt produces Cry proteins, which are activated in the alkaline environment of insect guts when ingested. Once activated, these proteins bind to specific receptors in the gut cells, creating pores in cell membranes, disrupting gut function, and causing cell lysis, paralysis, and eventual death of the insect. Transgenic crops expressing a single Bt gene have been engineered to produce Cry proteins, which remain inactive in the plant and only become active when ingested by targeted pests. This ensures that Bt proteins do not pose a threat to the plant or non-target organisms.

Since the introduction of Bt crops in 1996, this technology has been widely adopted, with Bt crops now used in 90% of field corn and upland cotton in the United States. The FDA has deemed Bt-expressing crops safe for consumption, and these crops are used in everyday products such as corn syrup and cotton-based textiles. While Bt crops have been effective in controlling pests like the corn borer, there is a growing need to extend this technology to other pest systems, such as plant-parasitic nematodes (Peferoen, 1997; Tabashnik and Carrière, 2017). One of the main challenges has been the nematodes' size-exclusion mechanisms, which were thought to prevent them from ingesting larger proteins such as Cry. However, *in vivo* studies have shown that incubating pre-infective juveniles of RKNs with Bt proteins like Cry5Ba2 and Cry6Aa2 exhibited toxicity to nematodes, opening new possibilities for nematode control (Zhang et al., 2012). More recent findings indicate that other nematode groups, such as cyst nematodes, can also ingest Bt proteins. For instance, Cry14Ab produced by Bt soybeans has been shown to reduce cyst nematode populations at harvest (Kahn et al., 2021).

To address the challenge of efficiently delivering Bt proteins to nematodes, Wang et al. (2024) investigated whether the plastids—specifically leucoplasts—can be exploited as a novel delivery system (Wang et al., 2024). Plastids, which include chloroplasts, leucoplasts, and etioplasts, play a vital role in photosynthesis and the biosynthesis of metabolites. Wang et al. (2024) demonstrated that the leucoplasts, which are primarily found in plant roots, accumulate in RKN-induced galls during infection and tend to break down into smaller vesicles, making them more palatable for nematodes to ingest. They then explored a novel approach by modifying the leucoplasts to express the Bt crystal protein Cry5Ba2, which effectively conferred resistance to RKNs.

A NOVEL APPROACH: UTILIZING THE LEUCOPLASTS FOR BT PROTEIN DELIVERY

To study leucoplast responses to RKN infection, Wang et al. (2024) used a transgenic *Arabidopsis* line with GFP-tagged leucoplasts (Wang et al., 2024). After 14 days post-inoculation with *M. incognita*, they observed increased GFP signals in galls, suggesting that nematodes modify infected root cells to accumulate leucoplasts at feeding sites. Plastid division genes were significantly upregulated in galls, confirming the increased number of leucoplasts in infected roots. Although the authors found that the number of leucoplasts increased in galls, nematodes feed on giant cells specifically embedded in galls. To determine leucoplast levels in giant cells, the authors performed immunoelectron microscopy analysis using antibodies to label leucoplasts (Wang et al., 2024). They found an increase in the number of leucoplasts in giant cells compared to uninfected roots and non-giant cells within galls. Interestingly, these leucoplasts were concentrated near the vacuole and mitochondrion in giant cells (Wang et al., 2024). Further investigations revealed that leucoplasts degraded into smaller vesicle-like structures (SVLs) during later stages of nematode infection (21 and 28 days post-inoculation), causing GFP fluorescence to leak from the leucoplasts. Remarkably, these SVLs were small enough to be

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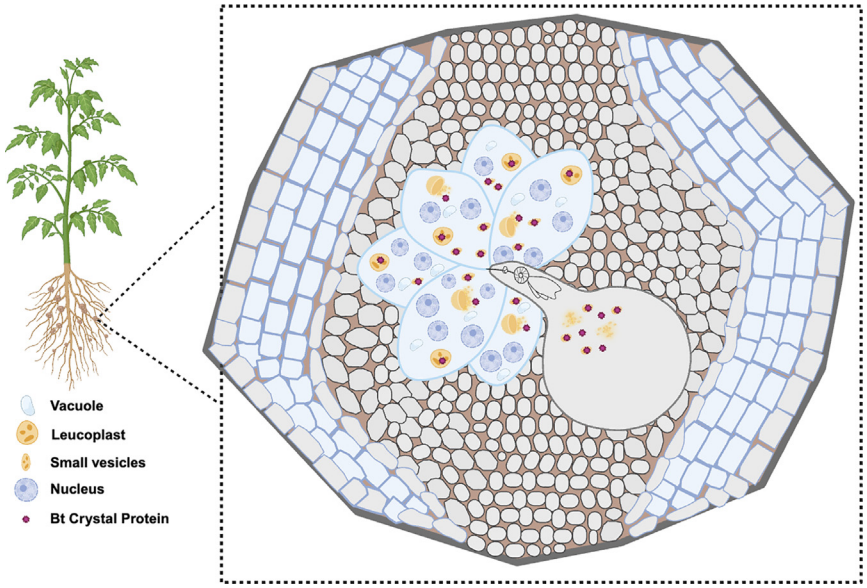

Figure 1. Consumption of Bt toxin delivered through the leucoplasts leads to nematode inhibition.
Schematic representation of leucoplast-targeted delivery of Cry5Ba2 via leucoplasts. In giant cells, the leucoplasts are degraded into small organelles that contain Cry5Ba2, which are subsequently ingested by nematodes. In addition, this degradation causes proteins, including Cry5Ba2, to leak into the cytosol, offering an alternative route for ingestion. Both mechanisms likely contribute to effective inhibition of nematodes. Created in BioRender (Blundell, 2021).

ingested by nematodes. Western blot analysis showed that proteins within these SVLs, including TIC110 (~110 kDa), were found inside adult female nematodes, confirming that nematodes can consume proteins delivered through leucoplast degradation (Figure 1) (Wang et al., 2024).

To efficiently express proteins in the leucoplasts of giant cells, the authors employed the transit peptide RecA1, which facilitates the transport of proteins into leucoplasts. They generated three types of tobacco lines: one expressing cytosolic GFP in the nuclear genome (Nt-nuGFP), another expressing GFP in the plastid genome (Nt-cpGFP), and a third expressing GFP in the nuclear genome but targeted specifically to leucoplasts using the RecA1 transit peptide (Nt-tpRecA1GFP). Confocal microscopy and western blotting demonstrated that the RecA1 peptide effectively directed GFP to leucoplasts, resulting in significantly higher GFP accumulation in Nt-tpRecA1GFP lines compared to both Nt-nuGFP and Nt-cpGFP lines (Wang et al., 2024).

The authors further generated transgenic plants expressing the Bt nematicidal protein Cry5Ba2 in cytosol or leucoplasts via the RecA1 transit peptide. A control group expressed GFP in the leucoplasts. When these lines were tested with *M. incognita*, both tobacco and tomato plants expressing Cry5Ba2 in leucoplasts or the cytosol showed a significant reduction in the number of galls and egg masses compared to the control group at 35 days post-inoculation. Western blot analysis of nematodes dissected from transgenic tomato plants confirmed that Cry5Ba2 was indeed ingested by nematodes. Notably, the amount of Cry5Ba2 ingested was significantly higher in nematodes feeding on plants expressing Cry5Ba2 in leucoplasts compared to cytosolic expression. Additionally, a marked reduction in the number of eggs per gram of root was observed in leucoplast-expressing Cry5Ba2 plants compared to cytosolic-expressing plants at 50 days post-inoculation (Wang et al., 2024).

Recent studies have made it clear that the expression of Bt proteins in plants has the potential to offer alternative management strategies for nematodes. These findings challenge the traditional understanding of nematodes' size-exclusion limits, warranting further exploration (Kahn et al., 2021; Wang et al., 2024). This study proposes leveraging leucoplasts as an efficient delivery system for Bt proteins (Wang et al., 2024). To fully realize the potential of Bt proteins in nematode control, testing across a broader range of crops and nematode species, including migratory nematodes that do not induce feeding sites, is necessary. It would also be interesting to explore leucoplasts as a delivery system for other nematicidal proteins, broadening their application beyond Bt. This study marks a significant advancement in nematode management, introducing leucoplasts as a novel delivery system for Bt proteins. With further research, this strategy could lead to more sustainable and effective pest control solutions for growers, reducing reliance on chemical nematicides.

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DECLARATION OF GENERATIVE AI AND AI-ASSISTED TECHNOLOGIES IN THE WRITING PROCESS
During the preparation of this work, the authors used ChatGPT to check spelling and grammar errors. After using this tool/service, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

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