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Review

Next-Generation Insect-Resistant Plants: RNAi-Mediated Crop Protection

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Plant-mediated RNA interference (RNAi) shows great potential in crop protection. It relies on plants stably expressing double-stranded RNAs (dsRNAs) that target essential genes in pest insects. Practical application of this strategy is challenging because producing sufficient amounts of stable dsRNA in plants has proven to be difficult to achieve with conventional transgenesis. In addition, many insects do not respond to exogenously applied dsRNAs, either degrading them or failing to import them into the cytoplasm. We summarize recent progress in RNAi-mediated insect pest control and discuss factors determining its efficacy. Expressing dsRNA in chloroplasts overcomes many of the difficulties previously encountered. We also highlight remaining challenges and discuss the environmental and biosafety issues involved in the use of this technology in agriculture.

RNAi for the Control of Insect Pests

RNA interference (RNAi) is a basic mechanism possessed by most eukaryotes by which gene expression in cells is downregulated by dsRNA intermediates (Box 1). Since its discovery in the nematode *Caenorhabditis elegans*, for which the Nobel Prize was awarded in 2006, RNAi has proved to be a powerful experimental tool for determining gene functions. One of the most active areas of agricultural biotechnology is the development of transgenic crop plants that can protect themselves from herbivorous insects by expressing dsRNA [1]. When dsRNA targeting essential insect genes is ingested by pests feeding on the plant, downregulation of these genes by the RNA interference (RNAi) pathway may result in reduced growth or death of the pest. This approach could be beneficial in reducing dependence on chemical insecticides [2], as well as in combating resistance to chemical insecticides and protein toxins from *Bacillus thuringiensis* [3]. The possibilities for such plant-mediated RNAi as well as its potential pitfalls (Box 2) have been extensively reviewed (e.g., [1,3–6]).

The strategy behind successful plant-mediated RNAi is different from conventional pest control strategies based on chemical insecticides. For example, organophosphorus insecticides kill insects by rapidly inhibiting the enzyme acetylcholinesterase (AChE, encoded by the *Ace-1* gene) at neuronal synapses in the central nervous system. Depletion of the AChE protein at the synapse by plant-mediated RNAi targeting the *Ace-1* gene would require time for sufficient dsRNA to be ingested by the insect, imported into neurons, released into the neuronal

Trends

Plant-mediated RNAi that targets essential genes in insects and other pests is becoming a promising approach in crop protection.

Expression of dsRNA targeting insect genes can potentially provide crop protection without chemical pesticides and offers the additional advantages that no foreign protein is made and the number of target genes is nearly unlimited.

The length and amount of the dsRNA as well as its stability *in planta* and in the gut of the target insect are crucial factors determining the success of plant-mediated RNAi strategies.

High-level expression of long dsRNAs from the genome of the chloroplast represents a particularly promising strategy for efficient RNAi-mediated crop protection.

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cytoplasm, and converted into **small interfering RNAs** (siRNAs, see Glossary) for destruction of existing and newly transcribed *Ace-1* mRNA. The rate of AChE protein turnover would then dictate how soon a physiological effect of the absence of AChE would occur in the insect. The fact that dsRNA must be ingested necessarily entails some feeding damage by the crop, and the delay in the mode of action must increase this damage. However, the great advantage of RNAi is that, in principle, any gene or genes in the insect can be targeted, and the vast majority of these have no commercially available insecticidal inhibitor (Figure 1).

An excellent example is provided by the transgenic maize developed by Monsanto for control of the western corn rootworm (WCR, *Diabrotica virgifera*; **Coleoptera**). This insect is susceptible to dsRNA administered in artificial diet, and this enabled the screening of a large number of dsRNAs for their effectiveness in feeding inhibition or mortality [7]. One of the most potent was directed against Snf7, an essential protein in vacuolar sorting [8]. So far no insecticide has been developed that interferes with the essential process of vacuolar sorting. Transgenic maize expressing *Snf7* dsRNA was found to protect maize roots from feeding damage by the subterranean WCR larvae, which escape chemical insecticide treatments. Because *Snf7* dsRNA alone takes a long time to kill WCR larvae, it will be deployed in combination with the faster-acting Cry3B insecticidal protein toxin from *B. thuringiensis*.

Another approach is to target an insect enzyme that interacts with a toxic chemical made by the plant to protect itself from insect herbivory. Gossypol is a polyphenolic compound of cotton that has many deleterious effects on animals, but the cotton bollworm *Helicoverpa armigera* (**Lepidoptera**) can tolerate moderate concentrations. Dietary gossypol induces expression of the cotton bollworm cytochrome P450, CYP6AE14. Under the assumption that the CYP6AE14 enzyme detoxifies gossypol, cotton was transformed with a construct targeting 469 nucleotides (nt) of the *CYP6AE14* transcript, and bollworm larvae consuming the transgenic cotton leaves had suppressed growth relative to those eating non-transgenic leaves [9]. Additional experiments showed that dsRNA directed against *CYP6AE14* only suppresses growth when gossypol is present in the diet, pointing to a specific interaction [9].

Another example is provided by nicotine, a neurotoxin made by species of tobacco. The tobacco hornworm *Manduca sexta* (Lepidoptera) can tolerate high nicotine concentrations. Larvae even exhale nicotine through their spiracles, deterring spider predation. Dietary nicotine induces the cytochrome P450 gene *CYP6B46* in *M. sexta*. *Nicotiana attenuata* was transformed with a construct expressing dsRNA targeting 300 nt of the *M. sexta* gene for CYP6B46. *M. sexta* larvae consuming the transformed tobacco were more susceptible to spider predation because they exhaled less nicotine [10]. One advantage of this approach is that a weak RNAi response may still be effective when it interacts with a plant chemical defense. However, the outcome depends crucially on the nature of the insect–plant interaction and may not be generally useful.

Differential Response to RNAi by Insects

Despite several attempts to apply this approach to other crops and insects [11–14], one challenge facing the successful implementation of plant-mediated RNAi for pest control is that many insects do not respond well to externally administered dsRNA (Box 2 and Figure 1). Lepidoptera (butterflies and moths) seem to be especially problematic, as discussed in a comprehensive comparison of experimental successes and failures [15]. Analysis of the growing number of insect genome sequences suggests that all possess the intracellular RNAi core machinery, but lack the **RNA-dependent RNA polymerases** (RdRPs) such as those found in nematodes that enable systemic RNAi [16]. Therefore, experimental studies have focused on perturbing genes affecting the stability and cellular import of dsRNA. The most recent comprehensive example used an **RNAi-of-RNAi approach** in a cell line of the Colorado potato beetle *Leptinotarsa decemlineata* (CPB; Coleoptera), which exhibits robust gene