Engineering chloroplasts for insect pest control

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Approximately half of the roughly 1 million insect species identified to date are herbivorous and constantly cause significant damage to crops. Despite substantial efforts, including the application of billions of pounds of chemical insecticides (1) arthropod pests cause major annual crop losses globally, especially in developing countries (2, 3), and these yield losses are projected to increase by 10 to 25% per degree of global mean surface warming (4). Therefore, safeguarding global food security requires us to develop innovative, effective, environmentally friendly crop protection strategies. Emerging approaches in crop protection have targeted chloroplasts as potential sites for "transplastomic" modifications that effectively protect plants from herbivorous insects.

Chloroplasts are derived from cyanobacteria and contain their own genomes (plastid DNA), providing opportunities for precise transgene insertion by homologous recombination (HR), which remains challenging for nuclear genes (5). Unlike the nuclear genome, chloroplast genomes lack epigenetic effects and are present in high copy numbers in plant cells. Chloroplasts thus offer the potential for extraordinarily high levels of expression of introduced genes.

Taking advantage of this potential for high transgene expression, the first application of transplastomic technology for pest control was the expression of the *Bacillus thuringiensis cry1A(c)* gene, which encodes an insecticidal protein, in tobacco (*Nicotiana tabacum*) chloroplasts. The transplastomic tobacco plants accumulated large amounts of cry1A(c), up to 3 to 5% of total soluble protein, and exhibited strong resistance to several insects (6). In addition to tobacco, transplastomic soybean (*Glycine max*) (7) and poplar (*Populus*) (8) plants expressing the *cry* gene also showed vigorous insecticidal activity.

Another emerging approach for pest control uses RNA interference (RNAi), a regulatory mechanism present in almost all eukaryotic organisms in which double-stranded RNA (dsRNA) generates small interfering RNAs (siRNAs) that mediate the destruction of messenger RNA (mRNA) with complementary sequences, thereby silencing the gene of interest (9). When dsRNA targeting an essential insect gene is expressed in plants and absorbed by insects via intestinal uptake following feeding, RNAi suppresses the expression of the targeted gene, resulting in impaired growth and death of the pest. Mao et al. (10) and Baum et al. (11) first described RNAi-based control strategies for insect pests using RNAs expressed from transgenes integrated into the nuclear genome.

The efficient uptake of long dsRNAs or hairpin RNAs (hpRNAs) is required for RNAi in insects. However, dsRNA expressed in the nucleus may be processed into siRNA by the plant's endogenous RNAi machinery, reducing its effect when fed to insects, and thus, it may be insufficient for protection in the field (12). Plant chloroplasts lack RNAi machinery, making them ideal for expressing dsRNA to achieve RNAi in insects. Indeed, Zhang et al. (13) provided a proof of concept for a pest control strategy by expressing dsRNA in potato (*Solanum tuberosum*) chloroplasts. Transplastomic potato plants expressing a long dsRNA targeting the *b-Actin* gene of *Leptinotarsa decemlineata* accumulated high levels of dsRNA in leaves, which induced much more potent RNAi in insects compared with its nuclear-transformed counterparts. Chloroplast-mediated RNAi also had significant effects on *Helicoverpa armigera* (14) and *Manduca sexta* (15). These target insects have chewing mouthparts that grind and consume solid plant tissues, thus releasing the dsRNA.

Agricultural insect pests have evolved diverse feeding modes to adapt to various food sources. In PNAS, Wu et al. (16) extend chloroplast-expressed dsRNA to control a nonchewing insect, western flower thrip (WFT; *Frankliniella occidentalis*), a destructive pest and virus vector that feeds on a wide range of outdoor crops and greenhouse vegetable and flower crops (Fig. 1). WFTs possess piercing-sucking mouthparts that they use to grasp plant cells and suck out the cellular contents. Damaged plant cells collapse, directly damaging the plants. In addition, WFT transmits many viruses that cause plant disease, including tomato spotted wilt virus that causes an annual loss worldwide of over $1 billion (17). Chemical control of WFT requires repeated foliar spraying using equipment that produces tiny droplets to secure good coverage and penetration into plant parts where thrips feed. Because most WFTs pupate in the soil, pesticides must also be applied to the ground. The development of resistance to major pesticides also makes WFTs challenging to control using chemical methods (18).

Thrips consume the contents of plant cells, including chloroplasts, suggesting that transplastomic dsRNA could be an efficient control strategy. To confirm that WFTs can ingest dsRNA in chloroplasts, WFT insects were fed an artificial diet, wild-type (WT) plants, and transplastomic tobacco plants expressing dsRNA targeting the *NADH (Nicotinamide adenine dinucleotide) dehydrogenase ubiquinone flavoprotein 2* gene of *H. armigera*. RT-PCR using RNA extracted from WFTs showed that the insects sucked up the chloroplast-expressed dsRNA upon feeding on the leaves of transplastomic plants.

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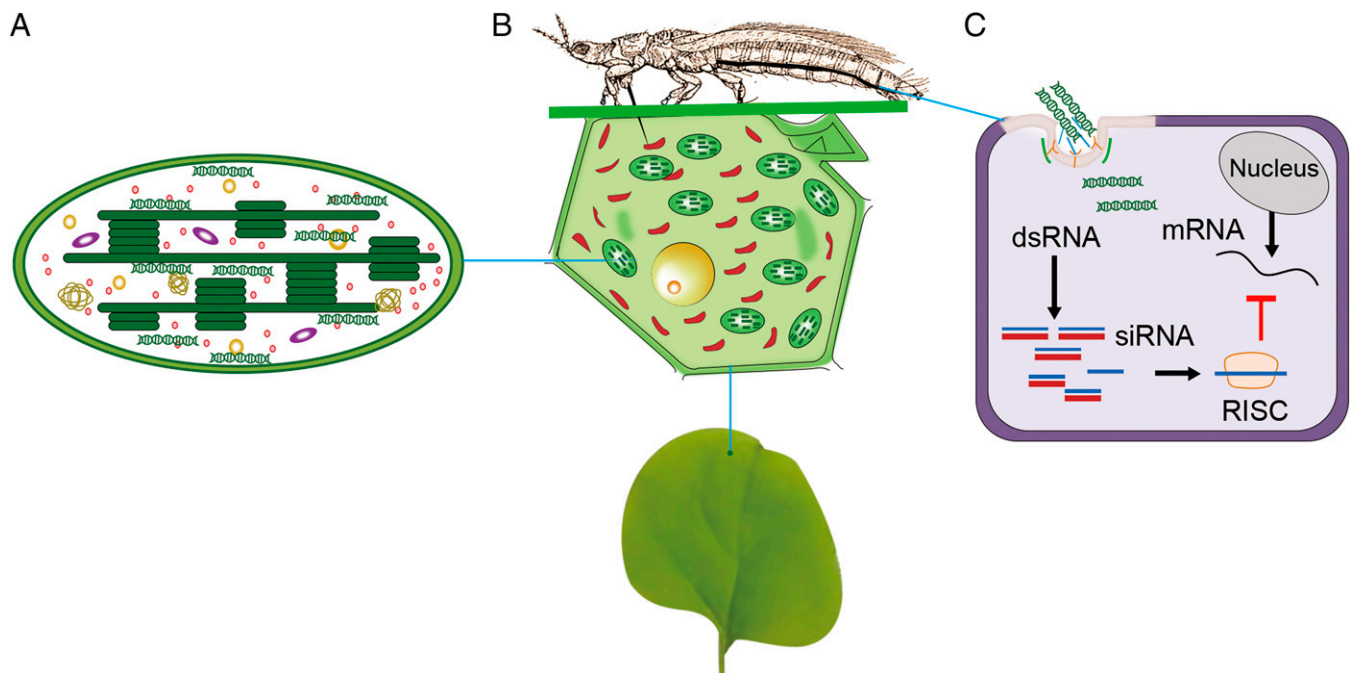
Fig. 1. Expression of dsRNA in chloroplasts for RNAi-mediated insect pest control. (*A*) Chloroplasts genetically engineered to express insect-specific long dsRNA at a high level. Chloroplasts do not process dsRNA into siRNA. (*B*) The insect (here a thrip) feeds on the chloroplast-transformed plants and ingests the cell contents, including chloroplasts that contain the dsRNAs. (*C*) dsRNAs are taken up by the insect gut cells and are processed into siRNAs to target and silence insect mRNAs, causing thrips mortality. RISC, RNA-induced silencing compex.

Next, Wu et al. (16) developed constructs harboring dsRNAs and hpRNAs targeting four essential genes of WFT: *ACT*, *TUB*, *VAT*, and *SNF*. These constructs were transformed into the chloroplast genome or nuclear genome to generate transplastomic and nuclear transgenic plants, respectively. The dsRNAs were estimated to make up ∼0.4 to 1% of total RNA levels in the transplastomic plants, and hpRNAs made up ∼0.4 to 0.8% of total RNA levels in these plants. Surprisingly, the dsRNA and hpRNA levels in the transplastomic lines were four orders of magnitude higher than those in the nuclear transgenic plants. On the third day of feeding, all four genes were significantly suppressed in WFT feeding on transplastomic plants expressing dsRNA or hpRNA compared with the WT and control. By contrast, WFT feeding on most nuclear transgenic lines had no significant effect on target gene expression in the insects.

The authors evaluated the WFT resistance levels of transplastomic and nuclear transgenic plants by performing insect bioassays and examining plant symptoms. They observed significantly higher mortality in larvae feeding on the leaves of transplastomic lines compared with WT plants. The insect resistance was much stronger in the transplastomic vs. nuclear transgenic lines. Analysis of leaf damage caused by WFTs verified the resistance levels of these lines. Twenty first-instar larvae were allowed to feed on the leaves for 4 d, and the damage was observed. WFTs caused substantially more severe damage to the leaves of WT and control plants compared with transplastomic plants. Analysis of the phenotypes of whole seedlings confirmed that the stronger resistance of transplastomic plants provided better protection against WFTs, likely due to the high levels of dsRNA and hpRNA produced in chloroplasts.

Gene constructs encoding intron-spliced RNA with a hairpin structure can increase RNAi efficiency (19). Transplastomic *N. benthamiana* plants expressing hpRNAs targeting the acetylcholine-sterase gene of *H. armigera* showed strong resistance against insect herbivory. Chloroplast-expressed dsRNA and hpRNA efficiently suppressed the targeted genes and caused high insect mortality. However, the hpRNA constructs caused chloroplast genome instability, suggesting that dsRNA cassettes are preferable for transplastomic RNAi. In addition to WFTs, many other insects in the order Thysanoptera are pests of commercial and food crops; the best known are *Stenchaetothrips biformis*, *Scirtothrips dorsalis*, *Thrips alliorum*, and *Haplothrips tritici*. Thrips are among the fastest-growing invasive species globally and are difficult to control using pesticides. The exciting research of Wu et al. (16) thus provides a promising strategy to control thrips and perhaps, other nonchewing insect pests.

One challenge in using transplastomic RNAi approaches is expanding the host range. Chloroplast transformation has been reported in over 20 flowering plants. However, to date, fewer than 10 species have been used to reproducibly generate homoplastic offspring. Most plants that have been successfully used for chloroplast engineering are in the Solanaceae, including tobacco, tomato (*S. lycopersicum*), potato, eggplant (*S. melongena*), and pepper (*Capsicum annuum*). Extending transplastomic RNAi approaches to cereals would improve our ability to control serious pests. Fundamental research into chloroplast biology and breakthroughs in chloroplast transformation technology should lead to the successful chloroplast transformation of recalcitrant cereals.

Researchers are using various approaches to improve chloroplast engineering in cereals and other plants. In general, the leaves of cereals cannot be cultivated and regenerated.

Instead, calli are dedifferentiated from embryos or young panicles and maintained on medium containing 2,4-dichlor-ophenoxyacetic acid. Deletions in Plastid DNA can occur during tissue culture (20). Plastids in callus cells may not be competent for foreign DNA integration. Approaches such as regulating the expression of morphogenic genes (21) may enhance the regeneration ability of leaves and mesophyll protoplasts and improve the competence of chloroplasts in cereals. Biolistics has long been the only reproducible method for DNA delivery into chloroplasts. However, a novel strategy for delivering plasmid DNA into chloroplasts via nanoparticles was recently introduced (22). A chloroplast signal peptide can guide nanomaterials loaded with chemicals into chloroplasts (23). Minisynplastomes could provide an alternative engineering platform for delivering foreign DNA into chloroplasts (24). Incorporating these latest DNA delivery tools will improve the ability and efficiency of stable chloroplast transformation in additional crops.

HR is a crucial step for the integration of alien DNA into the chloroplast genome. The HR frequency depends on the sequence carried in the foreign DNA identical to the target integration site. A comprehensive understanding of the intergenic sequences in the chloroplast genome and their diversity in crops will facilitate the development of efficient chloroplast engineering systems. The selection procedure for screening homoplastic plants has not changed much over the past decades. Although several antibiotic resistance genes have been tried, the streptomycin 30-adenylyltransferase gene (aadA) remains the most used selectable marker gene for chloroplast transformation (25). A recent study revealed that null mutations in *ACC2*, encoding a plastid-targeted acetyl-coenzyme A carboxylase, cause hypersensitivity to spectinomycin. The efficiency of chloroplast transformation in the *acc2* background increased ~100-fold vs. the control, providing valuable information for chloroplast engineering in recalcitrant crops (26). Along with the use of insect resistance genes (27) and nuclear transformation, chloroplast engineering may yield new opportunities to help safeguard food security, human health, and the agroecosystem.

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