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Efficient control of western flower thrips by plastid-mediated RNA interference

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Plastid-mediated RNA interference (PM-RNAi) has emerged as a promising strategy for pest control. Expression from the plastid genome of stable double-stranded RNAs (dsRNAs) targeted against essential insect genes can effectively control some herbivorous beetles, but little is known about the efficacy of the transplastomic approach in other groups of pest insects, especially nonchewing insects that do not consume large amounts of leaf material. Here we have investigated the susceptibility of the western flower thrip (WFT, *Frankliniella occidentalis*), a notorious pest in greenhouses and open fields, to PM-RNAi. We show that WFTs ingest chloroplasts and take up plastid-expressed dsRNAs. We generated a series of transplastomic tobacco plants expressing dsRNAs and hairpin RNAs (hpRNAs) targeted against four essential WFT genes. Unexpectedly, we discovered plastid genome instability in transplastomic plants expressing hpRNAs, suggesting that dsRNA cassettes are preferable over hpRNA cassettes when designing PM-RNAi strategies. Feeding studies revealed that, unlike nuclear transgenic plants, transplastomic plants induced a potent RNAi response in WFTs, causing efficient suppression of the targeted genes and high insect mortality. Our study extends the application range of PM-RNAi technology to an important group of nonchewing insects, reveals design principles for the construction of dsRNA-expressing transplastomic plants, and provides an efficient approach to control one of the toughest insect pests in agriculture and horticulture.

RNAi | plastid transformation | Thysanoptera | *Nicotiana tabacum* | pest control

Significance

Thrips are a group of piercing-sucking pest insects that do massive damage in agriculture and horticulture. The western flower thrip is a particularly notorious pest that has spread all over the world and is extremely difficult to control. In this work, we have shown that upon feeding, thrips take up substantial quantities of chloroplast RNA. When we expressed from the chloroplast genome long double-stranded RNAs that are targeted against essential thrip genes, the RNAs induced a potent RNA interference response and efficiently killed the insects. Our study demonstrates that an important group of nonchewing pest insects can be targeted by plant-mediated RNA interference and provides an efficient weapon to control thrips and other sucking plant pests.

RNA interference (RNAi) technology is considered a promising alternative to chemical pesticides. It achieves pest control by the delivery of double-stranded RNAs (dsRNAs) targeted against essential insect genes. Plant-mediated RNAi for pest control has been achieved through nuclear or plastid (chloroplast) transformation. Nuclear transgenic plants expressing dsRNA against insect essential genes have been proven to confer a limited level of protection from several insects, including the cotton bollworm (*Helicoverpa armigera*) (1, 2), the western corn rootworm (*Diabrotica virgifera*) (3, 4), the brown planthopper (*Nilaparvata lugens*) (5), the peach aphid (*Myzus persicae*) (6), and the whitefly (*Bemisia tabaci*) (7). However, the resistance levels attainable by nuclear transgenic strategies are often insufficient for practical application.

By contrast, expression of dsRNA from the plastid genome (transplastomic technology) led to strongly increased accumulation levels of dsRNA, due to absence of an RNAi machinery from plastids (8). It has been demonstrated that RNAi provoked by small interfering RNAs (siRNAs) is less efficient than the RNAi response triggered by long dsRNAs (9). The oral delivery of intact dsRNA was also shown to be a more effective RNAi trigger than nucleus-encoded dsRNAs (8, 10). Plastid-mediated RNA interference (PM-RNAi) showed a promising effect in controlling some chewing insects such as the Colorado potato beetle (*Leptinotarsa decemlineata*) (8), the corn earworm (*Helicoverpa zea*) (11), and the cotton bollworm (*H. armigera*) (10).

The western flower thrip (WFT, *Frankliniella occidentalis*; Thysanoptera) is an extremely polyphagous insect that attacks a wide range of host plant species in both greenhouses and open fields (12). With the international trade of agricultural products, it has spread over the world and become a global pest that causes severe economic damage (13). The insect's cryptic habits such as embedding eggs in plant tissues, and larvae and adults hiding under bracts or in buds, make the WFT extremely difficult to control (14). Moreover, the WFT has evolved resistance to most pesticides, thus further increasing the challenges with controlling the pest (15). Besides the damage caused directly through feeding and oviposition, the transmission of viruses through the WFT aggravates the damage and the economic losses caused (16).

Genes encoding homologs of the core components of the RNAi machinery are present in the genome of the WFT (17). Delivery of dsRNA through an artificial diet (18), microinjection (19), and symbionts (20) has successfully been exploited to

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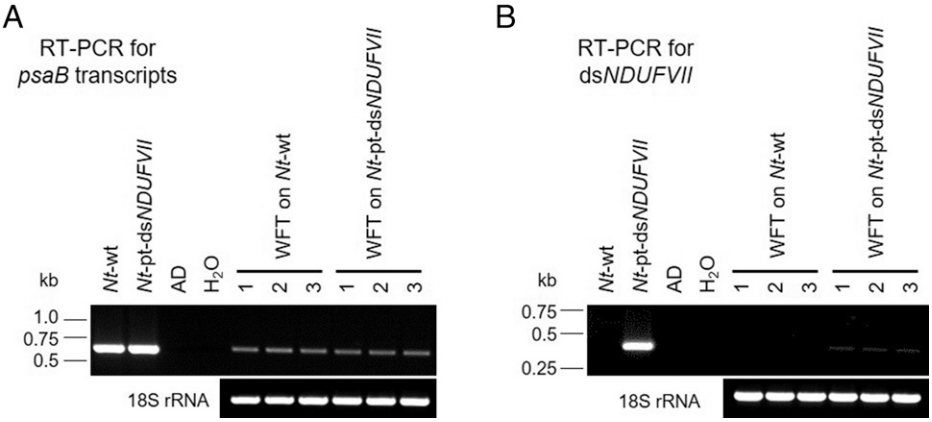

Fig. 1. Detection of transcripts derived from genes of the host plant in western flower thrips. (*A*) Detection of transcripts from the tobacco plastid genome-encoded *psaB* gene in WFTs fed on wild-type and transplastomic *Nt*-pt-ds*NDUFVII* tobacco plants by RT-PCR analysis. Total RNA samples from tobacco plants (*Nt*-wt and *Nt*-pt-ds*NDUFVII*) served as positive controls; total RNA extracted from WFT feeding on an artificial diet (AD) served as negative control. H_2O: water control. (*B*) Detection of ds*NDUFVII* RNA in WFTs fed on wild-type and *Nt*-pt-ds*NDUFVII* tobacco plants by RT-PCR analysis. Total RNA isolated from *Nt*-pt-ds*NDUFVII* plants served as positive control; total RNA samples from wild-type tobacco (*Nt*-wt) and from WFT feeding on AD served as negative controls. Insects fed on *Nt*-pt-ds*NDUFVII* were transferred to wild-type plants before collection to exclude contamination with cells of *Nt*-pt-ds*NDUFVII* plants. Transcripts from the WFT *18S rRNA* gene were amplified with gene-specific primers as internal control.

provoke an RNAi response in the WFT. These findings indicated that the plant-mediated RNAi technology could be a suitable strategy for WFT control. Additionally, the WFT is a piercing-sucking insect that causes extensive damage by emptying the cytoplasmic contents of plant cells (21), raising the possibility that, upon feeding, the WFT takes up the dsRNA accumulated in plastids. In this way, an efficient RNAi response could be triggered, and PM-RNAi could provide an efficient strategy to fight the WFT.

In this study, transplastomic and nuclear transgenic tobacco plants have been generated to express dsRNAs or hairpin RNAs (hpRNAs) that target four essential genes (*ACT*, *TUB*, *VAT*, and *SNF*) of the WFT. All four target genes were expressed as both dsRNA and hpRNA from the plastid genome. *Actin* (*ACT*) was chosen as a target gene, because suppression of this gene in *L. decemlineata* led to 100% lethality in larvae (8). The *tubulin* (*TUB*) gene was reported as a successful target gene for WFT control by symbiont-mediated RNAi (20). Delivery of dsRNA targeted against *vacuolar ATPase catalytic subunit B* (*VAT*) by artificial diet or microinjection also led to increased mortality in the WFT (18, 19). Finally, the endosomal sorting complex required for transport III subunit *Snf7* (*SNF*) is widely used as a target gene in pest control (22, 23). Besides the choice of the target gene, the length and molecular conformation of the dsRNA are also important factors that determine the efficiency of the RNAi response in insects. Although dsRNA (8, 11) and hpRNA (10) have been exploited to induce RNAi via plastid transformation, which molecular conformation initiates a stronger RNAi response and/or has a higher stability in plastids is currently unknown. We report that hpRNA-expressing transplastomic plants are genetically less stable than dsRNA-expression transplastomic plants, probably due to nonreciprocal homologous recombination and microhomology-mediated sequence deletion. However, both types of transplastomic plants were well protected from WFTs and performed much better than nuclear transgenic plants.

Results

Accessibility of Plastid Content to WFTs. To determine whether WFTs can access dsRNA expressed from the plastid genome and contained within the chloroplast compartment upon feeding, RT-PCR assays were conducted to examine the presence of chloroplast messenger RNA (mRNA) and plastid-expressed dsRNA in feeding insects. To this end, a transplastomic tobacco line (*Nt*-pt-ds*NDUFVII*) expressing dsRNA targeted against the nicotinamide adenine dinucleotide (NADH) dehydrogenase ubiquinone flavoprotein 2 (*NDUFVII*) gene of *H. armigera* (24) was offered to the insects, followed by extraction of RNA from WFT feeding on wild-type (WT) control plants and transplastomic tobacco plants. Subsequently, cDNA was synthesized and tested for the presence of mRNA from the plastid-encoded *psaB* gene (encoding the B subunit of the photosystem I reaction center) and the ds*NDUFVII* transgene residing in the plastid genome of *Nt*-pt-ds*NDUFVII* plants. Interestingly, both *psaB* and ds*NDUFVII* transcripts were detected in insects feeding on *Nt*-pt-ds*NDUFVII* plants, whereas insects feeding on wild-type plants contained only the *psaB* RNA (Fig. 1). No RT-PCR signals for *psaB* or ds*NDUFVII* were detected in WFTs after feeding on an artificial diet, as expected. These results demonstrate that plastid-expressed dsRNAs are sucked up by WFTs upon feeding on leaf material, thus raising the attractive possibility to control WFTs through PM-RNAi.

Generation and Molecular Characterization of Transplastomic and Nuclear Transgenic Tobacco Plants Expressing Anti-WFT dsRNAs. Four essential insect genes were selected as RNAi targets: The genes for the cytoskeletal proteins actin (*ACT*) and tubulin (*TUB*), the gene for the catalytic subunit B of vacuolar ATPase (*VAT*), and the gene encoding endosomal sorting complex required for transport III subunit SNF7 (*SNF*). For all four target genes, two types of plastid transformation vectors were designed to 1) produce dsRNA by annealing of single-stranded sense and antisense transcripts and 2) synthesize self-complementary RNA (hpRNA). The dsRNA is generated in plastids by transcription from two convergent copies of the tobacco ribosomal RNA operon promoter (*Prrn*), while the hpRNA is produced by transcribing two inverted repeats of the transgene from a single-copy *Prrn* promoter and stabilized with the *rrnB* terminator (*TrrnB*) from *Escherichia coli* (Fig. 2*A*). The eight plastid transformation constructs were introduced into the tobacco plastid genome by particle gun–mediated (biolistic) transformation. For each construct, several

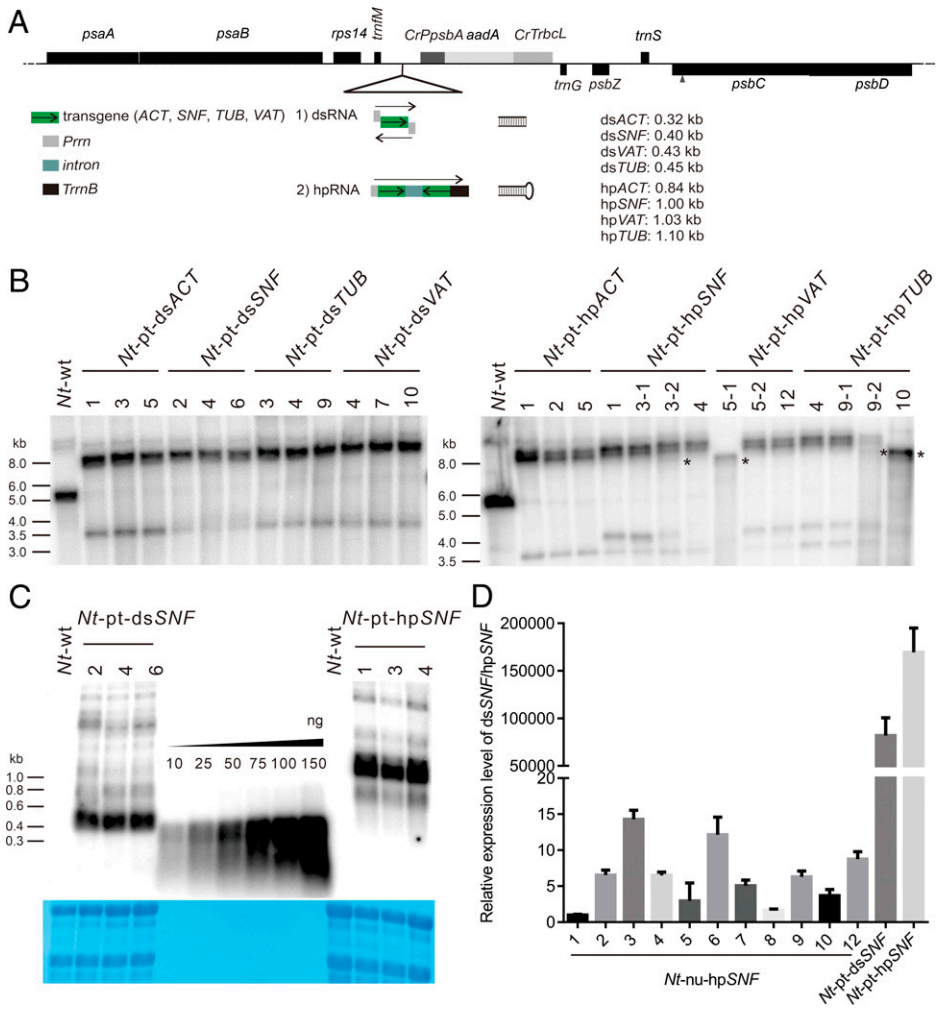

Fig. 2. Generation of transplastomic and nuclear transgenic tobacco plants expressing RNAi construct targeted against WFTs. (*A*) Physical map of plastid transformation vectors. The cassettes designed to produce dsRNAs and hpRNAs are schematically depicted *Below* the map, along with the expected structures and sizes of the RNA strands produced. The dsRNA is generated by two convergent 16S rRNA promoters (*Prrn*) from tobacco. The hpRNA is transcribed by *Prrn* and stabilized by the *rrnB* terminator (*TrrnB*) from *E. coli*. The selectable marker gene *aadA* is driven by the *psbA* promoter (*PpsbA*) and the 3′ untranslated region (3′ UTR) of the *rbcL* gene (*TrbcL*) from *Chlamydomonas reinhardtii*. Genes above the line are transcribed from *Left* to *Right*, *Below* the line are transcribed in the opposite direction. (*B*) Southern blot analysis of transplastomic tobacco plants. Total DNA was digested with the restriction enzyme *Eco*NI and hybridized to a radiolabeled *psaB*-specific probe. The asterisks indicate the abnormal hybridizing band that arises from deletion of the hpRNA cassette by microhomology-mediated illegitimate recombination. See text (*Plastid Genome Instability Caused by Insertion of hpRNA Cassettes*) for details. (*C*) Analysis of the accumulation levels of ds*SNF* and hp*SNF* in three independent transplastomic lines by northern blotting. The methylene blue–stained membrane prior to blotting is shown *Below* the blot as loading control. (*D*) Analysis of the relative expression levels of the hp*SNF* RNA in nuclear transgenic lines by qRT-PCR in comparison to dsRNA and hpRNA expression levels in plastids. The tobacco *ACTIN* gene was used as an internal standard. Data are shown as means ± SD (three biological replicates).

independent transplastomic lines were obtained, two to three of which were selected for further analysis. Southern blot analyses were conducted to assess the segregation of wild-type and transgenic plastid genome copies, and the homoplastomic state of transplastomic lines was evidenced by virtual absence of a hybridization signal for the ∼5.6-kb fragment diagnostic of the wild-type genome and exclusive presence of the larger fragments corresponding to the transformed genomes (∼7.9 kb for *Nt*-pt-ds*ACT*, ∼8.0 kb for *Nt*-pt-ds*SNF* and *Nt*-pt-ds*VAT*, ∼8.1 kb for *Nt*-pt-ds*TUB*, ∼8.5 kb for *Nt*-pt-hp*ACT*, ∼8.6 kb for *Nt*-pt-hp*SNF*, and ∼8.7 kb for *Nt*-pt-hp*VAT* and *Nt*-pt-hp*TUB*) (Fig. 2*B* and *SI Appendix*, Fig. S1*A*). Homoplasmy was ultimately confirmed by seed assays and lack of segregation of the antibiotic resistance conferred by the selectable marker gene *aadA* (encoding aminoglycoside-3″-adenylyltransferase and conferring spectinomycin resistance) in the next generation (*SI Appendix*, Fig. S2*A*) (25, 26).

Next, the expression levels of dsRNAs and hpRNAs in our transplastomic lines were examined by northern blot analyses (Fig. 2*C* and *SI Appendix*, Fig. S3). For semiquantitative assessment of dsRNA and hpRNA accumulation levels in plastids, a dilution series of in vitro synthesized dsRNA was included in all RNA gel blots. Based on relative signal intensities, the accumulation levels of ds*ACT*, ds*SNF*, ds*VAT*, and ds*TUB* were estimated to be about 1%, 0.4%, 0.6%, and 0.8% of total RNA, respectively. The expression levels of the hpRNAs were comparably high, with hp*ACT*, hp*SNF*, hp*VAT*, and hp*TUB* accumulating to ∼0.4%, 0.6%, 0.8%, and 0.8% of total RNA, respectively. All transplastomic plants displayed wild-type–like phenotypes and grew normally (*SI Appendix*, Figs. S2 and S4).

To be able to compare thrip protection levels in transplastomic and nuclear transgenic plants, an RNAi vector for hpRNA expression from the nuclear genome was constructed (construct pMT100 targeting the WFT *SNF* gene) (*SI Appendix*, Fig. S1*B*) and introduced into tobacco by *Agrobacterium*-mediated transformation. Twelve independent nuclear transformed lines were selected, and the hp*SNF* expression levels in these transgenic lines were determined by qRT-PCR (Fig. 2*D*). Two lines displaying

high expression levels were chosen for subsequent experiments. dsRNA and hpRNA accumulation in the transplastomic lines is approximately four orders of magnitude higher than in the nuclear transgenic plants (Fig. 2*D*). This finding is consistent with the rapid breakdown of the dsRNA expressed in the nucleus by the plant's own RNAi machinery. Similar to the transplastomic lines, the nuclear transgenic lines exhibited wild-type–like phenotypes and showed no growth defects or developmental aberrations (*SI Appendix*, Figs. S2 and S4).

Plastid Genome Instability Caused by Insertion of hpRNA Cassettes. We noticed that, unexpectedly, all the transplastomic lines expressing hpRNA constructs showed a double band for the transgenic plastid genome in Southern blot analyses (Fig. 2 *B*, *Right*). As the insect gene fragments are present in two copies in the hpRNA lines, they can potentially undergo nonreciprocal homologous recombination upon formation of dimeric plastid genomes. Due to the presence of many copies of the plastid genome in a single organelle (27) and the highly active homologous recombination system operating in plastids (28), plastid genomes regularly form dimers and higher-order multimers (29). Indeed, the sizes of the hybridization signals observed in Southern blots were in agreement with dimer/multimer formation by nonreciprocal homologous recombination, in addition to the presence of monomers and dimers/multimers formed by reciprocal homologous recombination (*SI Appendix*, Figs. S1*A* and S5). We, therefore, conclude that two monomeric plastid genomes stochastically undergo reciprocal and nonreciprocal homologous recombination, thus leading to the presence of similar amounts of two distinct genome conformations (Figs. 2*B* and 3*A*). In order to directly confirm this hypothesis, additional Southern blot analyses were performed with the restriction enzyme *Bst*Z17I that would resolve the two conformations more clearly. Indeed, the sizes of the resulting hybridization signals (Fig. 3*B*) matched the sizes predicted to occur upon presence of a mixed population of plastid genome molecules, with an ∼50% representation of the nonreciprocal recombination product (Fig. 3*B* and *SI Appendix*, Fig. S6).

The Southern blot analyses (Fig. 2*B*) also revealed unexpectedly small hybridizing fragments in several transplastomic lines transformed with hpRNA constructs. For example, the transplastomic lines *Nt*-pt-hp*VAT*5-1 and *Nt*-pt-hp*TUB*10 showed only a single smaller-than-expected band (Fig. 2*B*), and no hp*VAT* and hp*TUB* transcripts were detectable (*SI Appendix*, Fig. S3). The absence of hpRNA expression in these transplastomic lines led us to assume that deletion of the transgene had occurred. To test this hypothesis, PCR analyses (with primers rps14-F and aadA-R) (*SI Appendix*, Table S1) were conducted to determine structure and sequence of the hpRNA expression cassette (Fig. 3*C* and *SI Appendix*, Fig. S7*A*). Two other transplastomic lines, *Nt*-pt-hp*SNF*3-2 and *Nt*-pt-hp*TUB*9-2, were also included in these experiments, because they also showed similar smaller hybridization signals in Southern blots, besides the band of the expected size (Fig. 2*B*). The size of the amplification products obtained (Fig. 3*C* and *SI Appendix*, Fig. S7*A*) suggested that in all four lines, ∼1 kb of the transgene cassette was lost, which roughly corresponds to the size of the entire hairpin sequence (compare Fig. 2*A*). Sequencing of the PCR products confirmed deletion of (most of) the hairpin sequence in *Nt*-pt-hp*SNF*3-2, *Nt*-pt-hp*VAT*5-1, *Nt*-pt-hp*TUB*9-2, and *Nt*-pt-hp*TUB*10. Interestingly, inspection of the breakpoints of the deletions revealed that, in all four lines, microhomologies were present, suggesting that the deletions could result from microhomology-mediated illegitimate recombination events (Fig. 3*D* and *SI Appendix*, Fig. S7*B*).

Assessment of the Resistance of Transplastomic and Nuclear Transgenic Plants to Western Flower Thrips. To evaluate the WFT resistance levels of transplastomic and nuclear transgenic plants, insect bioassays with WFT larvae were conducted. The survival curves of first-instar WFT larvae feeding on detached leaves from control plants (*Nt*-wt and *Nt*-pt-ds*NDUFVII*), transplastomic (*Nt*-pt-ds*SNF* and *Nt*-pt-hp*SNF*), and nuclear transgenic plants (*Nt*-nu-hp*SNF*) were recorded for 6 consecutive days. Compared to the nuclear transgenic plants, the transplastomic plants induced substantially higher mortality, with nearly all larvae being dead after 6 d (Fig. 4*A*). Pairwise statistical analysis of survival curves between all groups showed that, as expected, there was no significant difference between wild-type plants and transplastomic control plants (*Nt*-wt vs. *Nt*-pt-ds*NDUFVII*, $P = 0.721$). Compared to wild-type plants, both types of transplastomic lines (*Nt*-pt-ds*SNF* and *Nt*-pt-hp*SNF*) showed strong resistance to WFT (*Nt*-wt vs. *Nt*-pt-ds*SNF*, $P < 0.001$ and *Nt*-wt vs. *Nt*-pt-hp*SNF*, $P < 0.001$). By contrast, one of the nuclear transgenic lines tested (*Nt*-nu-hp*SNF*4) had no significant effect on WFT (*Nt*-wt vs. *Nt*-nu-hp*SNF*4, $P = 0.200$). The other nuclear transgenic plant line (*Nt*-nu-ds*SNF*3), which had higher hpRNA expression levels (Fig. 2*D*), was significantly different from the wild-type plants (*Nt*-wt vs. *Nt*-nu-hp*SNF*3, $P < 0.01$), but its WFT resistance level was still far below that of any of the transplastomic lines (Fig. 4*A*). When the two types of transplastomic lines (ds*SNF* and hp*SNF*) were compared, there was no significant difference in their level of WFT resistance (*Nt*-pt-ds*SNF* vs. *Nt*-pt-hp*SNF*, $P = 0.153$), suggesting similar effects of dsRNA and hpRNA on WFT control.

Next, we examined the transplastomic plants expressing dsRNA or hpRNA constructs targeted against the other three WFT genes (*ACT*, *TUB*, and *VAT*). The results of the insect bioassays showed that these transplastomic plants caused similarly high mortality in WFT larvae as the transplastomic plants targeting *SNF* (*SI Appendix*, Fig. S8*A*). Therefore, all four WFT genes tested represent efficient RNAi targets for WFT control, and transplastomic expression of either dsRNA or hpRNA provokes an efficient RNAi response in WFTs.

We next analyzed the expression levels of the RNAi target genes in WFT feeding on wild-type, transplastomic, and nuclear transgenic plants after 5 d by qRT-PCR (Fig. 4*B* and *SI Appendix*, Fig. S8*B*). As expected, feeding on control plants (*Nt*-pt-ds*NDUFVII*) did not lead to any changes in target gene expression at the mRNA level in the insects (*Nt*-wt vs. *Nt*-pt-ds*NDUFVII*). By contrast, feeding on transplastomic plants expressing WFT sequences resulted in suppressed expression of all targeted genes (Fig. 4*B* and *SI Appendix*, Fig. S8*B*). Consistent with the absence of insect mortality (Fig. 4*A*), WFT feeding on the nuclear transgenic line *Nt*-nu-hp*SNF*4 had no significant effect on *SNF* gene expression in the insects (Fig. 4*B*; *Nt*-wt vs. *Nt*-nu-hp*SNF*4, $P = 0.161$), feeding on the transplastomic lines (*Nt*-pt-ds*SNF* and *Nt*-pt-hp*SNF*) and the strongly expressing nuclear transgenic line (*Nt*-nu-hp*SNF*3), knocked down the expression of the *SNF* gene in the insect (*Nt*-wt vs. *Nt*-nu-hp*SNF*3, $P < 0.05$; *Nt*-wt vs. *Nt*-pt-ds*SNF*, $P < 0.001$; *Nt*-wt vs. *Nt*-pt-hp*SNF*, $P < 0.001$). In agreement with the mortality data, suppression of *SNF* expression was more pronounced in WFTs fed on the transplastomic lines than in thrips fed on the nuclear transgenic line *Nt*-nu-hp*SNF*3 (Fig. 4*B*). To determine the onset of the RNAi effect, *SNF* expression levels in thrips were analyzed between day 2 and

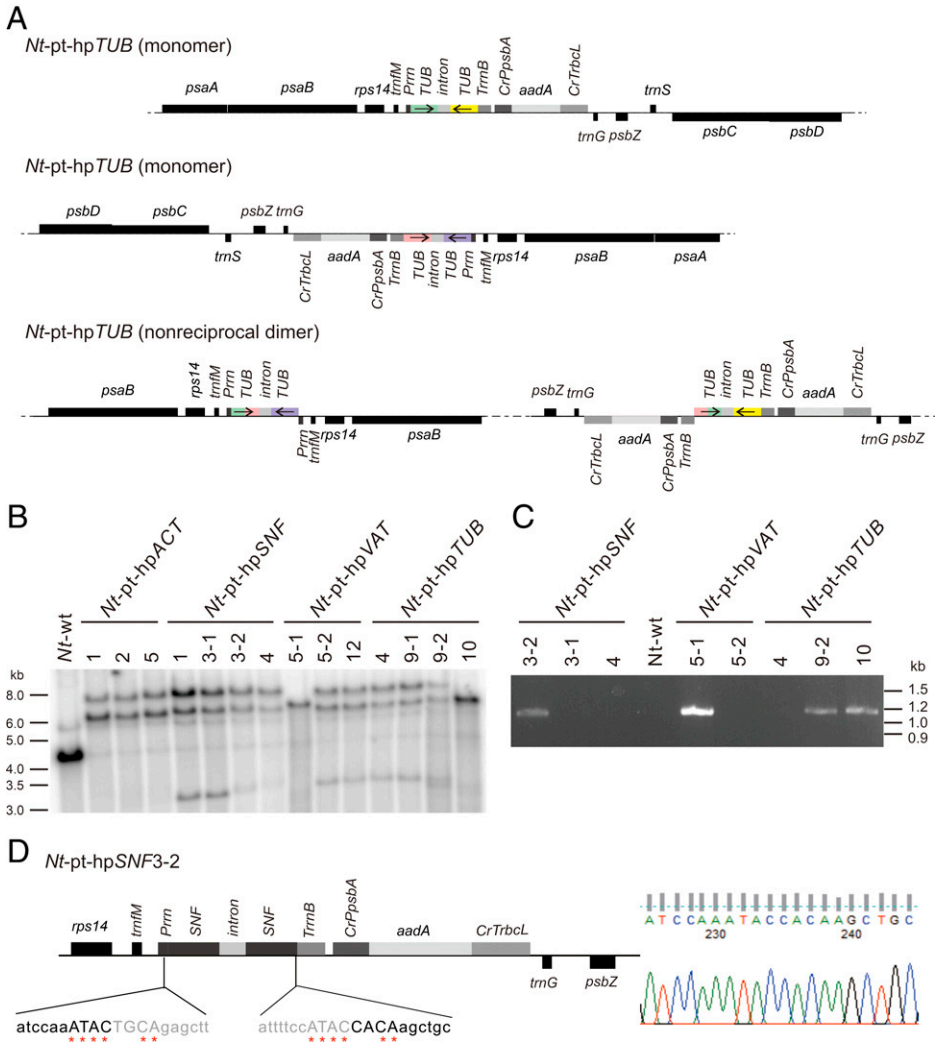

Fig. 3. Verification of nonreciprocal homologous recombination and microhomology-mediated sequence deletion in transplastomic lines expressing hpRNA constructs. (*A*) Schematic map illustrating the process of nonreciprocal homologous recombination via the hpRNA cassette (shown for *Nt*-pt-hp*TUB* as an example). Two plastid genomes form a dimeric genome by recombination between the sense *TUB* sequence in one genome copy (*Upper* map) and the antisense *TUB* sequence in another genome copy (*Lower* map). The resulting new *TUB* hairpin cassette is now flanked by two promoters (*Lower* map) or, alternatively, by two terminators. (*B*) Southern blot analysis of transplastomic tobacco plants expressing hpRNA constructs. Total DNA was digested with *Bst*Z17I and hybridized to a radiolabeled *psaB* probe. (*C*) PCR assays reveal presence of a (partially) deleted hpRNA cassette. A pair of flanking primers (rps14-F and aadA-R) was chosen to amplify the hpRNA cassette. Note that an amplification product is obtained only from lines harboring deletions but not from the control plants (*Nt*-pt-hp*SNF*3-1, *Nt*-pt-hp*SNF*4, *Nt*-pt-hp*VAT*5-2, and *Nt*-pt-hp*TUB*4), due to inability of the DNA polymerase to amplify the large inverted repeats of the intact hpRNA cassette. (*D*) Detection of microhomologies at the breakpoints of the deletion. As an example, the sequencing information in an *Nt*-pt-hp*SNF* plant is shown (which lost most of the hpRNA transgene). The location of the breakpoints is indicated in the physical map, and identical nucleotides are marked with red asterisks. The sequence stretches exhibiting microhomology are shown in capital letters. Gray characters indicate deleted sequences; black characters indicate retained sequences. The part of the sequence chromatogram encompassing the breakpoints is also shown. For additional examples, see *SI Appendix*, Fig. S5.

day 5 of feeding on nuclear transgenic and transplastomic plants. The results revealed that the transplastomic plants trigger a significant RNAi response as early as 3 d after feeding (*SI Appendix*, Fig. S8*C*).

To directly visualize the protective effects of transplastomic expression of anti-thrips constructs, we also examined the leaf damage caused by WFTs (Fig. 4 *C* and *D* and *SI Appendix*, Fig. S9*A*). To this end, 20 first-instar larvae were encased in a Petri dish and allowed to feed on detached leaves for 4 d. The damaged leaves were then photographed and the damaged area was quantified by image analysis. Both visual inspection of leaves and image quantification confirmed that WFTs caused substantially more damage to wild-type and control plants (*Nt*-wt and *Nt*-pt-ds*NDUFVII*) than to transplastomic plants (*Nt*-pt-ds*SNF* and *Nt*-pt-hp*SNF*). There was no significant difference between wild-type plants and the nuclear transgenic line, *Nt*-nu-hp*SNF*4, while the best-performing nuclear transgenic line *Nt*-nu-hp*SNF*3 showed less damage than the wild type, but more damage than the transplastomic plants. Insect bioassays with whole seedlings also confirmed that transplastomic plants conferred superior protection from WFT (Fig. 4*E* and *SI Appendix*, Fig. S9*B*). This finding is consistent with the uptake of much larger amounts of dsRNA by the insects from transplastomic plants compared to nuclear transgenic plants (*SI Appendix*, Fig. S10).

Discussion

WFTs greatly benefited from international trade and have become an invasive pest in most parts of the world. Due to its unique lifestyle and feeding behavior, controlling and efficiently managing the WFT in greenhouses or open fields is extremely

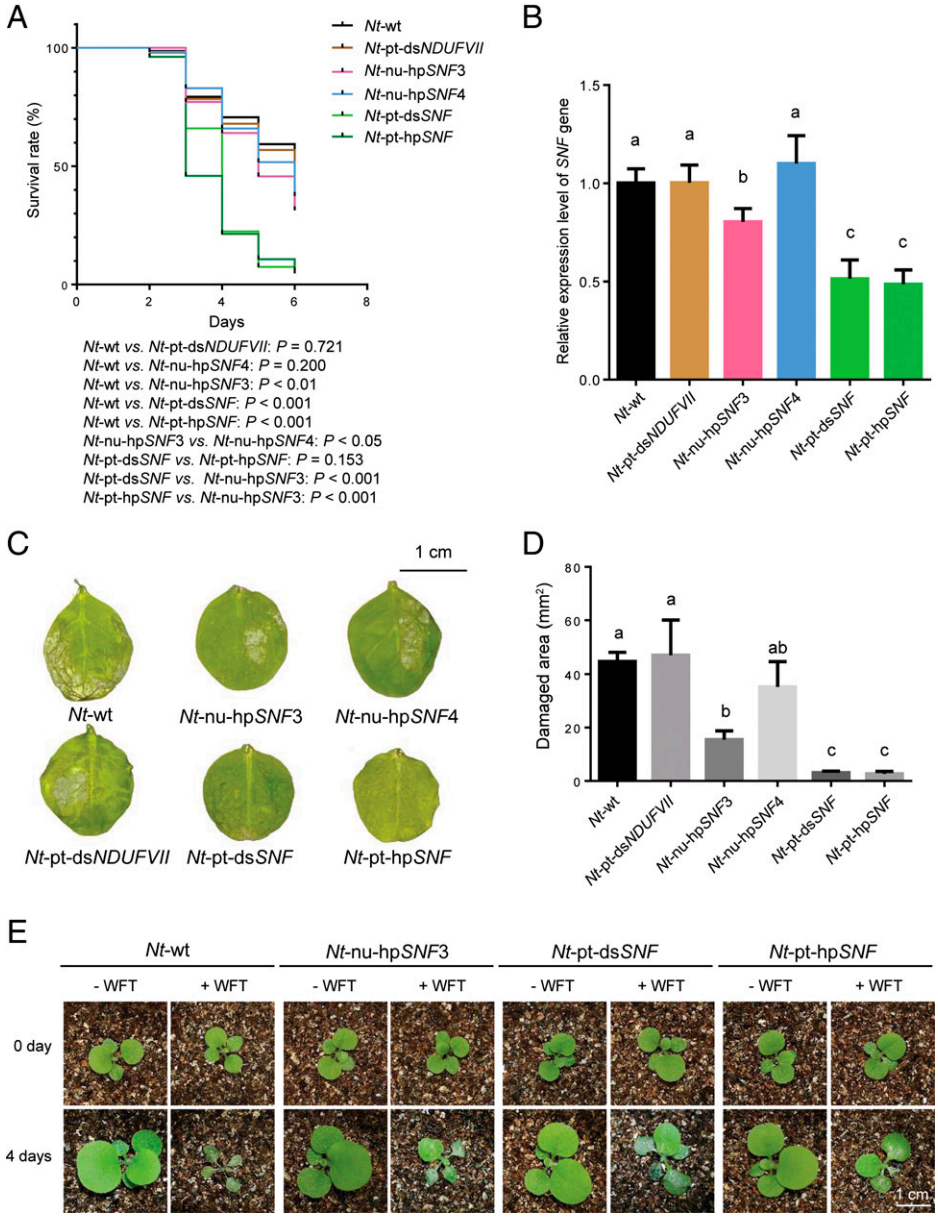

Fig. 4. Feeding assays of WFTs with transplastomic and nuclear transgenic tobacco plants. (*A*) Survivorship of first-instar larvae upon feeding on detached leaves of wild-type, transplastomic, and nuclear transgenic tobacco plants. *Nt*-pt-ds*NDUFVII* is a transplastomic control line expressing ds*NDUFVII* targeted against the cotton bollworm. The *P* values for each pair of plant lines were calculated and are given at the *Bottom*. (*B*) Relative expression levels of the *SNF* gene in WFTs fed on wild-type, transplastomic, and nuclear transgenic tobacco plants at day 5 were determined by qRT-PCR. The data represent means ± SD (three biological replicates with 20 insects per sample). Significant differences to the wild-type control were identified by ANOVA ($P < 0.05$). (*C*) Comparison of feeding damage by WFT first-instar larvae after 4 d (three biological replicates with 20 insects per sample). (*D*) Leaf area consumed by WFT larvae after 4 d in the experiment shown in *C*. The data represent means ± SD. Significant differences to the wild-type control were identified by ANOVA ($P < 0.05$). (*E*) Comparison of feeding damage in 3-wk-old plants caused by WFT adults after 4 d of feeding (three biological replicates with 12 insects per sample).

challenging (30). In this work, we have tested the idea that PM-RNAi (31) can be used for WFT control. We have shown that the transplastomic technology can protect plants from the WFT. Moreover, comparing transplastomic plants with nuclear transgenic plants expressing the same RNAi construct, the transplastomic approach proved superior and led to much better protection from the WFT. This is due to 1) higher dsRNA/hpRNA accumulation levels in plastids and 2) absence from plastids of an RNAi machinery that would cleave dsRNAs into siRNAs that have much lower insecticidal activity than long intact dsRNAs (8, 32).

Our data show that both dsRNAs and hpRNAs derived from four different thrip genes could be expressed to high levels from the plastid genome. Although the same *Prrn* promotor was used for all four transgenes and for the generation of both dsRNA and hpRNA, there was variation in RNA accumulation levels. The length of the gene fragments and possibly their GC content may contribute to the observed differences in RNA accumulation (Fig. 2*C* and *SI Appendix*, Fig. S3). There is no strict correlation between the type of RNA expressed (dsRNA or hpRNA) and the level of protection from WFTs or between the RNA accumulation level and the protection level, although higher RNA levels tend to be beneficial. For example, the ds*ACT*-expressing lines (accumulating 1% dsRNA of the total cellular RNA) caused a higher mortality of WFT larvae than the hp*ACT*-expressing lines (accumulating 0.4% hpRNA of the total RNA) (*SI Appendix*, Fig. S2). Similarly, the hp*VAT*-expressing lines (accumulating 0.8% hpRNA of the total RNA)

conferred stronger protection against WFTs than the ds*VAT*-expressing lines (accumulating 0.6% dsRNA of the total RNA). However, overall, the differences between the transplastomic lines targeting the same insect gene were relatively minor, indicating that plastid-expressed dsRNA and hpRNA both act as effective triggers of RNAi responses in WFT.

Clearly, some initial damage is done by the insects even to the transplastomic plants. This is because the thrips feed on the transplastomic plants for up to 6 d before they die (Fig. 4*A*). Thus, although there is also less damage to the leaves (Fig. 4 *C*–*E*), long-term plant protection occurs predominantly by preventing reproduction of the thrips.

Although plastid-expressed dsRNA and hpRNA show similar efficiencies with respect to WFT control, our work reported here has revealed that hpRNA constructs can negatively impact plastid genome stability in two ways. First, the presence of two copies of the RNAi target sequence triggers nonreciprocal homologous recombination events that lead to rearranged transgene cassettes (Fig. 3*A*). Second, the hpRNA-expressing lines proved to be prone to deletion of the transgene fragment (Fig. 3*D* and *SI Appendix*, Fig. S7*B*) by microhomology-mediated illegitimate recombination. Microhomologies of only very few nucleotides are known to cause deletions in nearly all biological systems, including bacteria (33, 34), the eukaryotic nucleus (35), and also in chloroplasts (36–38). A possible mechanistic explanation for the observed instability in the hpRNA constructs but not the dsRNA constructs could be that the hpRNA cassette constantly undergoes flip-flop recombination between the sense and the antisense sequence of the transgene, and therefore, may be prone to recombination errors that result from the recombination machinery occasionally acting on microhomologies. Taken together, our data suggest that transgenes present as long inverted DNA repeats are unstable in plastid genomes and, if possible, should be avoided.

Our work shows that PM-RNAi provides an effective approach to control WFTs. It is well known that the susceptibility to environmental RNAi varies greatly in different groups of insects. The presence of double-stranded ribonucleases in the digestive tract, endosomal entrapment, variations in the components of the core RNAi machinery, and inadequate immune stimulation are some of the factors that potentially limit the RNAi efficiency in some insects (31, 39). We searched the currently available genomic data (assembly accession: GCF_000697945.2) for WFTs for the presence of genes related to RNAi. All core components of the RNAi machinery, and proteins related to dsRNA internalization (SID1-like protein) could be identified in the WFT genome. Phylogenetic trees were constructed to assess the similarity of Dicer, AGO, SID1, and Staufen among insects (*SI Appendix*, Fig. S11). The Staufen protein of WFTs showed high similarity with its homologs from Coleoptera and may improve the dsRNA processing efficiency, as recently reported for *Thrips tabaci* (23). As expected from data in other insects, no gene for an RNA-dependent RNA polymerase (RdRP) could be identified in the WFT genome, suggesting absence of secondary siRNA synthesis. The lack of secondary siRNA production likely explains the superiority of the transplastomic approach in that it causes a need for continuous provision of large amounts of dsRNAs to trigger efficient gene silencing in WFT.

It is becoming increasingly clear that the efficiency of protection from herbivorous insects that is conferred by the transplastomic technology is highly related to the feeding habits of the insects. For example, dsRNA expressed in plastids remains inaccessible to sap-sucking insects such as the whitefly *B. tabaci*, and consequently, transplastomic plants expressing dsRNA targeted against the *B. tabaci ACTIN* gene do not affect this insect (40). The mouthparts of Thysanoptera (thrips) comprise two stylets, the mandibular stylet and the maxillary stylets (41). Upon feeding, the paired maxillary stylets interlock and form a single tube, serving as a food canal, while the single mandibular stylet is used to pierce substrates (Fig. 5). Due to this feeding habits, the WFT is able to access the contents of both epidermal and mesophyll cells (Fig. 1) (42). Through the tube

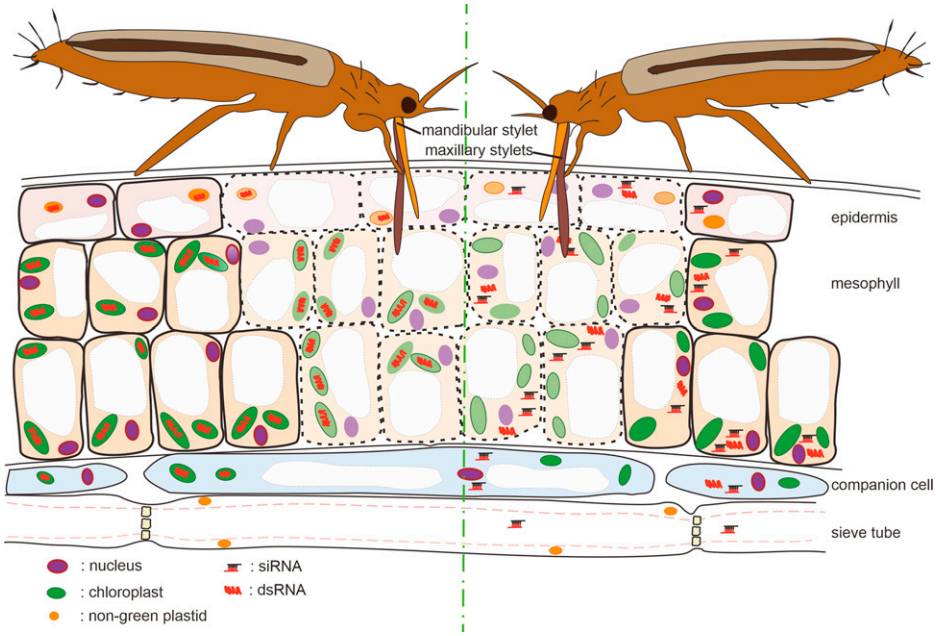

Fig. 5. Schematic model of the mechanisms involved in WFT feeding on transplastomic and nuclear transgenic plants expressing dsRNAs against essential thrip genes. The insects pierce plant cells with their mandibular stylet and extend the paired maxillary stylets to suck up cellular content. Long dsRNAs accumulate to high levels in transplastomic plants (*Left*) and are encapsulated in the chloroplast (and nongreen plastids of epidermal cells). Thrips can ingest entire chloroplasts (21), but it is also possible that some chloroplasts are damaged upon feeding and their stroma leaks into the cytosol. In nuclear transgenic plants, dsRNA is processed into siRNAs by the plant's own RNAi machinery, which results in low insecticidal activity (31). The cell walls at WFT feeding sites are marked with dotted lines. The model is not drawn to scale.

formed by their maxillary stylets, thrips have been shown to ingest entire chloroplasts (21), thus likely facilitating the efficient uptake of dsRNA/hpRNA expressed in plastids (Fig. 5).

In summary, we have shown that PM-RNAi offers an effective strategy for WFT control and is superior to dsRNA expression from the nuclear genome. Our study demonstrates that an important group of nonchewing insects can be targeted by PM-RNAi, thus extending the technology to Thysanoptera and potentially other sucking plant pests. Our work also reveals important design criteria for transplastomic RNAi constructs and suggests that hpRNAs cassettes can cause genome instability and, therefore, are less preferable than dsRNA cassettes flanked by convergent promoters.

Materials and Methods

Plant Material. Kidney bean (*Phaseolus vulgaris*) pods were disinfected with bleach (0.65% active chlorine) and used to raise WFT colonies. Tobacco plants were grown axenically on Murashige and Skoog (MS) medium (43) supplemented with 3% (wt/vol) sucrose. Transplastomic and nuclear transgenic tobacco (*Nicotiana tabacum* L. cv. Petit Havana) lines were generated by biolistic plastid transformation and *Agrobacterium*-mediated nuclear transformation technologies, respectively. Transplastomic line *Nt*-pt-ds*NDUFVII* expresses a 364-bp dsRNA (ds*NDUFVII*; nucleotide positions +197 to +560) targeted against NADH dehydrogenase ubiquinone flavoprotein 2 (*NDUFVII*) of the mitochondrial complex I of the cotton bollworm *H. armigera*, an efficient RNAi target gene for inhibiting larval growth of *H. armigera* (24). dsRNA expression is driven by two convergent 16S rRNA promoters, and the dsRNA-expression cassette was targeted to the *trnfM*/*trnG* region in the plastid genome (Fig. 2*A*). *Nt*-nu-hp*NDUFVII* is a transgenic control line that expresses a hairpin construct (containing the same target sequence as the ds*NDUFVII* construct) from the nuclear genome. Expression is driven by the double 35S promoter from cauliflower mosaic virus, and the loop of the hairpin is formed by the first intron from the potato GA20-oxidase gene. The *NDUFVII* target sequence lacks homology to any gene in the WFT genome (44), making *Nt*-pt-ds*NDUFVII* an ideal material to determine the accessibility of plastid-encoded dsRNAs by the WFT and measure dsRNA uptake efficiency.

WFT Rearing. WFT colonies were reared in tube-shaped glass jars (4 L) with snap-on lids. A 10-cm diameter hole was cut in the lid and covered with a fine mesh to allow for ventilation. The insects were reared under controlled conditions of 26 ± 1 °C, 70 ± 10% humidity and a 14-h/10-h light/dark (L14:D10) photoperiod (45, 46). Female adults were allowed to oviposit on the bean pods for 24 h and then removed to collect an even-aged cohort of WFTs. The egg-containing bean pods were transferred to a fresh jar and placed in an incubator at 26 ± 1 °C with a L14:D10 photoperiod until nymphs hatched.

Vector Construction for Plant Transformation. Plastid transformation vectors were constructed based on the previously generated vectors pWW1 (47) and pYY12 (48). For construction of dsRNA-expressing vectors, plasmid pWW1 was digested with *Sac*I and *Sbf*I, and primers (ACT-F and ACT-R, SNF-F and SNF-R, TUB-F and TUB-R, VAT-F and VAT-R) (*SI Appendix*, Table S1) containing 20- or 21-nt homologous sequences with pWW1 were used to amplify the target gene fragments. The amplification products obtained were then inserted into the cut pWW1 with the help of the In-Fusion HD Cloning Kit (Clontech), generating the plastid dsRNA-expressing vectors pMT90 (expressing ds*ACT*), pMT91 (expressing ds*SNF*), pMT92 (expressing ds*TUB*), and pMT93 (expressing ds*VAT*), respectively. For construction of the hpRNA-expressing vector pMT94 (expressing hp*ACT*), the sense *ACT* DNA fragment was amplified with primer pair ACT-F1/ACT-R1 (*SI Appendix*, Table S1). Following PCR amplification of the *GA20* intron and the antisense *ACT* DNA fragment with primer pairs intron-F/intron-ACT-I-R and intron-ACT-A-F/intron-ACT-A-R (*SI Appendix*, Table S1), respectively, an overlap-extension PCR was conducted to fuse the *GA20* intron with the antisense *ACT* DNA fragment and introduce *Sal*I and *Not*I restriction sites. Finally, the *Pst*I/*Xho*I-excised sense *ACT* DNA fragment, the *Sal*I/*Not*I-excised fusion of the GA20 intron and the antisense *ACT* DNA fragment, and the *Pst*I/*Not*I-excised pYY12 (48) were ligated to generate vector pMT94. Plastid transformation vectors pMT95 (expressing hp*SNF*), pMT96 (expressing hp*VAT*), and pMT97 (expressing hp*TUB*) were generated using similar procedures as for pMT94, and assembled by ligation of three PCR fragments.

To generate the nuclear transformation vector pMT100, the excised hp*SNF* expression cassette was cloned as the *Pst*I/*Not*I fragment into vector pHW25 (49). The cloning steps for all transformation vectors were carried out in *E. coli* strain DH5α. PCR was performed with Phusion High-Fidelity DNA Polymerase (Thermo Fisher) following the manufacturer's instructions. All primers used in this study are listed in *SI Appendix*, Table S1.

Plastid and Nuclear Transformation. Tobacco plants for plastid transformation were grown under aseptic conditions on MS medium supplemented with 30 g/L sucrose until the seedling reached half the height of the Magenta box. Young leaves were collected and bombarded with the PDS1000/He particle delivery system equipped with a Hepta adaptor (Bio-Rad). Bombarded leaves were sliced into 5 mm × 5 mm pieces and placed onto RMOP (Regeneration Medium of Plants) medium containing 500 mg/L spectinomycin (50). For each construct, several independent transplastomic lines were subjected to additional rounds of regeneration on spectinomycin-containing medium to select for homoplasmy.

Nuclear transgenic plants were generated by *Agrobacterium*-mediated transformation (51). Leaves from 6-wk-old tobacco plants were infected with *Agrobacterium tumefaciens* (strain EHA105) containing vector pMT100. Infected explants were transferred to RMOP medium containing 100 mg/L kanamycin and 500 mg/L carbenicillin for selection. The hpRNA expression levels in nuclear transgenic tobacco plants were analyzed by qRT-PCR.

Rooted transplastomic and nuclear transgenic plants were transferred into soil and grown in a greenhouse for seed production.

Nucleic Acids Isolation and Gel Blot Analyses. Total DNA was extracted from fresh leaf samples by a cetyltrimethylammonium bromide (CTAB)-based method (52). For Southern blot analysis, samples of 5 μg total DNA were digested with appropriate restriction enzymes, separated by electrophoresis in 0.8% agarose gels, and transferred onto nylon membranes (Hybond-XL, GE Healthcare). A 550-bp PCR product covering part of the *psaB* coding region was used as a hybridization probe (53). For northern blot analysis, total cellular RNA was extracted from leaf samples of soil-grown tobacco plants using the peqGOLD TriFast reagent (Peqlab). After denaturation, RNA samples (of 5 μg total plant RNA) were separated by electrophoresis in 1% formaldehyde-containing agarose gels and blotted onto Hybond-N membranes (GE Healthcare). PCR products generated by amplification with gene-specific primers (*SI Appendix*, Table S1) were used as hybridization probes. Hybridizations were performed at 65 °C using standard protocols for Southern and northern blots. [α^{32}P]-dCTP–labeled probes were generated using the Multiprime DNA Labeling System (GE Healthcare).

In Vitro Synthesis of dsRNA. Synthesis of dsRNA was carried out with the HiScribe T7 Quick High Yield RNA Synthesis Kit (NEB) according to the manufacturer's instruction. The T7 promoter sequence (5′-GGATCCTAATACGACTCACTA-TAGG-3′) was added to the 5′ end of forward and reverse primers (*SI Appendix*, Table S1). The RNA yield was determined with a Nano Photometer (Implen) and the dsRNA was kept at −80 °C until further use.

Insect Bioassays. Even-aged WFTs were allowed to feed on wild-type, transplastomic, and nuclear transgenic plants. Detached leaves of 4-wk-old plants were harvested and placed onto a wet filter paper inside a Petri dish (6.5 cm diameter, 1 cm height). A total of 20 first-instar larvae were transferred onto the detached leaves with a fine brush (10 replicates for each line), and the number of dead insects was determined daily.

To assay the feeding damage, tests with both detached leaves and whole seedlings were performed. The plant material was offered to WFT larvae and adults, respectively; photos were taken after 4 d of feeding; and the damaged area was determined with ImageJ. For the damage assays conducted with whole seedlings, wild-type (*Nt*-wt), transplastomic (*Nt*-pt-ds*SNF* and *Nt*-pt-hp*SNF*), and nuclear transgenic plants (*Nt*-nu-hp*SNF3*) were grown for 3 wk (in vermiculite) and exposed to WFT adults (n = 12) for 4 d. Photos were taken on day 0 and day 4 of feeding.

qRT-PCR. Total RNA of WFTs was extracted with the RNAiso Plus reagent (Takara) following the manufacturer's protocol. After digestion with a gDNA digester (Yeasen), cDNA was synthesized using the RevertAid First-Strand cDNA Synthesis Kit

(Yeasen). The reaction mixture for PCR amplification consisted of 2 μL of cDNA, 0.25 μL of forward and reverse primers, and 5 μL TB Green Premix Ex Taq II (Takara) in a final 10-μL reaction volume. The relative expression levels of target genes were calculated by the $2^{-\Delta\Delta CT}$ method (54, 55). Gene expression levels in WFTs were measured by qRT-PCR between day 2 and day 5 of the bioassay. The elongation factor 1α (*EF1α*) was chosen as a reference gene (56).

Statistical Analysis. Prior to statistical analysis (SPSS software, version 19.0), the normality of data distribution was examined (Shapiro–Wilk test for normality; $P > 0.05$). The gene expression data (for nuclear transgenic plants and insects) and the quantified damaged leaf area were analyzed using one-way analysis of variance (ANOVA). The data are presented as means with the corresponding SD for all experiments. The survival curves were analyzed using the Kaplan–Meier method.

Data Availability. All study data are included in the article and/or *SI Appendix*.

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