**PEST CONTROL**

**Full crop protection from an insect pest by expression of long double-stranded RNAs in plastids**

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Double-stranded RNAs (dsRNAs) targeted against essential genes can trigger a lethal RNA interference (RNAi) response in insect pests. The application of this concept in plant protection is hampered by the presence of an endogenous plant RNAi pathway that processes dsRNAs into short interfering RNAs. We found that long dsRNAs can be stably produced in chloroplasts, a cellular compartment that appears to lack an RNAi machinery. When expressed from the chloroplast genome, dsRNAs accumulated to as much as 0.4% of the total cellular RNA. Transplastomic potato plants producing dsRNAs targeted against the β-actin gene of the Colorado potato beetle, a notorious agricultural pest, were protected from herbivory and were lethal to its larvae. Thus, chloroplast expression of long dsRNAs can provide crop protection without chemical pesticides.

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**SUPPORTING MATERIALS**

www.sciencemag.org/content/347/6225/988/suppl/DC1

Materials and Methods

Supplementary Text

Figs. S1 to S7

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References (53–58)

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25. Materials and methods are available as supplementary materials on Science Online.
38. The regression analyses applied to the full CMIP5 multimodel mean yields a scaling factor (“beta”) for Northern Hemisphere temperature changes that slightly exceeds unity (beta = 1.053 ± 0.0169), implying a real-world forced response that is slightly greater than that estimated by the CMIP5 multimodel mean. In contrast, North Atlantic mean temperatures yield a scaling factor slightly below unity (beta = 0.916 ± 0.0155), and North Pacific mean temperatures yield a scaling factor substantially below unity (beta = 0.650 ± 0.0380), suggesting that the CMIP5 multimodel mean substantially overestimates the amplitude of forced temperature changes over the North Pacific. Further details, including results for the two subsensembles (CMIP3-AIE and CMIP3-GISS), are available in the supplementary materials (table S3).
40. NMO = 0.35 AMO + 0.45 PMO for CMIP3-AIE; NMO = 0.42 AMO + 0.36 PMO for CMIP3-GISS; NMO = −0.06 AMO + 0.85 PMO for CMIP3-AIE; AMO and PMO regression coefficients are significant at the P << 0.05 level based on a one-sided test.

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All raw data, Matlab code, and results from our analysis are available at the supplementary website: www.meteo.psu.edu/holocene/ public_html/supplements/Science2015. We acknowledge the World Climate Research Programme’s Working Group on Coupled Modeling, which is responsible for CMIP, and we thank the climate modeling groups for producing and making available their model output. We thank K. Emanuel and G. Schmidt for helpful comments on earlier versions of the manuscript. B.A.S. acknowledges support by the U.S. National Science Foundation Atmospheric and Geospace Sciences–Postdoctoral Research Fellowships (AGS-PRF (AGS-1137750). Kaplan SST V2 data were provided by the NOAA/Office of Oceanic and Atmospheric Research/Earth System Research Laboratory Physical Sciences Division, Boulder, Colorado, USA: www.esrl.noaa.gov/psd. HadISST data were provided by the Met Office Hadley Centre: www.metoffice.gov.uk/hadobs. ERISST data were provided by NOAA: www.ncdc.noaa.gov/data-access/marineocean-data/extended-reconstructed-sea-surface-temperature-erst-v3.

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Double-stranded RNAs (dsRNAs) targeted against essential genes can trigger a lethal RNA interference (RNAi) response in insect pests. The application of this concept in plant protection is hampered by the presence of an endogenous plant RNAi pathway that processes dsRNAs into short interfering RNAs. We found that long dsRNAs can be stably produced in chloroplasts, a cellular compartment that appears to lack an RNAi machinery. When expressed from the chloroplast genome, dsRNAs accumulated to as much as 0.4% of the total cellular RNA. Transplastomic potato plants producing dsRNAs targeted against the β-actin gene of the Colorado potato beetle, a notorious agricultural pest, were protected from herbivory and were lethal to its larvae. Thus, chloroplast expression of long dsRNAs can provide crop protection without chemical pesticides.
As essential target genes, the CPB ACT and SHR genes were chosen. ACT encodes β-actin, an essential cytoskeletal protein, and SHR encodes Shrub (also known as Vps32 or Snf7), an essential subunit of a protein complex involved in membrane remodeling for vesicle transport. Disruption of these genes when the insects are fed in vitro synthesized dsRNAs induces mortality with high efficacy (3, 17). To test longer dsRNAs and test for a possible synergistic action, we also produced an ACT+SHR fusion gene. To confirm the activity of these dsRNAs in the beetles, we synthesized the dsRNAs (ACT, SHR, ACT+SHR fusion, and GFP as a control) by in vitro transcription, painted them onto young potato leaves, and fed the leaves to second-instar CPB larvae. All three insect gene–derived dsRNAs reduced larval growth (fig. S2). The ACT dsRNA was more effective than the SHIR dsRNA, and the ACT+SHR dsRNA was the least effective dsRNA (fig. S2), indicating that targeting two insect genes with the same dsRNA does not necessarily enhance insecticidal activity.

We first evaluated the three strategies for in vivo dsRNA production (ptDP, ptSL, and ptHP constructs; Fig. 1A and fig. S1C) with the ACT+SHR fusion gene in tobacco plants, because chloroplast transformation is relatively routine in this species. Transplastomic tobacco lines were produced by particle gun–mediated chloroplast transformation and purified to homoplasy by additional rounds of regeneration and selection. Transgene integration into the plastid genome by homologous recombination and elimination of all wild-type copies of the highly polyploid plastid genome were confirmed by restriction fragment length polymorphism analyses and inheritance assays (figs. S1E and S3A). All transplastomic lines (referred to as Nt-ptDP-ACT+SHR, Nt-ptSL-ACT+SHR, and Nt-ptHP-ACT+SHR) displayed no visible phenotype and were indistinguishable from wild-type plants, both under in vitro culture conditions and upon growth in the greenhouse (fig. S3, B and D), indicating that dsRNA expression in the chloroplast is phenotypically neutral.

To test whether dsRNAs stably accumulate in chloroplasts, we performed Northern blot analyses. The results revealed that all three types of expression constructs triggered production of substantial amounts of long dsRNAs (Fig. 1B), suggesting the absence of efficient dsRNA-degrading mechanisms from plastids. dsRNA accumulation levels in Nt-ptDP and Nt-ptSL plants were very similar, indicating that the terminal stem-loop structures added to the ptSL constructs do not increase dsRNA stability (Fig. 1, A and B). dsRNA accumulation levels in Nt-ptHP lines were even higher but included shorter-than-expected transcripts (Fig. 1B), possibly because the plastid RNA polymerase encounters difficulties transcribing sequences containing large inverted repeats. Therefore, we used the convergent promoter approach (ptDP constructs) for dsRNA expression in all subsequent experiments.

We next introduced the three target gene constructs (ACT, SHR, and ACT+SHR) into the ptDP cassette (Fig. 1A) into the plastid genome of potato (14) (see supplementary materials), the main host of CPB, and isolated homoplasmic transplastomic lines (St-ptDP-ACT, St-ptDP-SHR, and...
To be able to compare the level of protection from herbivory in transplastomic and nuclear transgenic plants, we introduced the identical transgenes (as hairpin constructs containing a spliceosomal intron that is posttranscriptionally removed) (15) into the nuclear genome by Agrobacterium-mediated transformation (St-nuHP; fig. S1D). Phenotypic analyses showed that all transplastomic and nuclear transgenic potato plants were indistinguishable from wild-type plants with regard to growth and tuber production (fig. S3, C and E, and figs. S4 and S5).

Northern blot analyses of transplastomic potato lines revealed that the accumulation levels of ACT dsRNAs were higher than those of SHR and ACT+SHR dsRNAs (fig. S1B). Comparison to a dilution series of in vitro synthesized RNA revealed dsRNA accumulation levels in leaves of ~0.4% of the total cellular RNA for ACT, ~0.05% for SHR, and ~0.1% for ACT+SHR (fig. S6). By contrast, hybridization signals in the nuclear transgenic

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Fig. 2. Feeding assays of CPB larvae on transplastomic and nuclear transgenic potato plants. (A) Survivorship of first-instar larvae upon feeding on detached leaves of wild-type, transplastomic, and nuclear transgenic potato plants. (B) Growth of surviving larvae. The weight of survivors was determined after 3, 5, 7, and 9 days of feeding. Data are means ± SD (n = 30). Significant differences to the wild-type control were identified by analysis of variance. *P < 0.05, **P < 0.01, ***P < 0.001. The best-performing nuclear transgenic lines were included in the assay (see figs. S7 to S10). For assays with additional transplastomic lines, see fig. S9. Note that the weight of survivors in the assays with the transplastomic plants expressing ACT dsRNA (St-ptDP-ACT21) could only be measured until day 3, because all larvae were already dead at day 5 [see (A)]. (C) Example of a bioassay with detached leaves of wild-type potato plants and nuclear transgenic and transplastomic leaves expressing ACT dsRNA. Leaves were exposed to first-instar CPB larvae, replaced with fresh young leaves every day, and the photograph was taken at day 3. Note that almost no visible damage is seen in St-ptDP-ACT21 leaves.

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Fig. 3. Consumption of detached leaves of potato plants by adult beetles, and survivorship of larvae upon feeding on whole plants. (A) Leaf area consumed by freshly emerged adult beetles fed on leaves of wild-type potato plants, nuclear transgenic plants, and transplastomic plants expressing ACT dsRNA. As an additional control, leaves painted with in vitro synthesized GFP-derived dsRNA were included. Data are mean ± SD (n = 24 for St-wt, n = 12 for all other lines). (B) Survivorship of second-instar CPB larvae after feeding on whole plants at day 5 (see fig. S12).
plants could be detected only upon overload-
ing of the gels and/or overexposure of the blots, consistent with efficient degradation of dsRNAs into siRNAs by the plant’s endogenous RNAi machinery (Fig. 1D and figs. S7 and S8A). The presence of siRNAs in nuclear transgenic plants and their absence from transplastomic plants were directly confirmed by Northern blot analyses (Fig. 1E).

Because CPB larvae and adults feed on leaves but not on belowground potato tubers, only the leaves need to be protected from herbivory. Non-photosynthetic tissues, such as tubers, express plastid to much lower levels than photosynthetic tissues, such as leaves (61, 17). Thus, despite whole-plant transformation, dsRNA production in tubers, where accumulation of transgene-derived RNA is unnecessary and perhaps undesired by the consumer, was below or near the detection limit (Fig. IC and fig. S8B).

Having established that long dsRNAs accumu-
late to high levels in leaves of transplastomic potato lines, we next tested whether dsRNA production in the chloroplast offers protection against CPB. To this end, the mortality of first-instar CPB larvae was determined upon feeding on detached leaves from wild-type, transplastomic, and nuclear transgenic potato plants for 9 consecutive days (Fig. 2A and fig. S9A). In addition, the weight of all surviving larvae was measured to follow their growth (Figs. 2B and C; figs. S9, B to D; and fig. S10). The bioassays revealed that all transplastomic potato plants induced high mortality (Figs. 2A and S6) and the high efficacy of in vitro synthe-
sized ACT dsRNA (fig. S2), the most potent insecticidal activity was conferred by the ACT dsRNA–expressing transplastomic plants that caused 100% mortality within 5 days. By contrast, none of the nuclear transgenic potato plants conferred any larval mortality (Figs. 2A and fig. S9A), in line with the earlier finding that short siRNAs fed to insects have only small effects or do not induce an RNAi response at all (3). However, all nuclear transgenic lines caused reduced growth of CPB larvae (Fig. 2B), presumably due to the small amounts of dsRNAs the plants accumulate (fig. 1D and figs. S7 and S8). While none of the CPB larvae survived feeding on transplastomic St-pdP-ACT plants, some of the larvae survived for 9 days on St-pdP-SHR and St-pdP-ACT leaves (fig. S11). However, these survivors suffered from very strong growth retardation (Fig. 2B and fig. S9, C and D).

To confirm that the killing of the CPB larvae by the transplastomic plants was due to induction of RNAi, we determined expression of the target genes in the larval gut after 3 days of feeding (i.e., when the larvae fed on the transplastomic plants were still alive). Already at this early stage, expression of β-actin and Shrub was suppressed in the insects (fig. S11, A and B). As expected on the basis of the mortality data (Fig. 2A), target gene suppression was strongest in larvae fed on St-pdP-ACT plants. Moreover, accumulation of ACT-derived siRNAs was detected in gut tissue of larvae fed with transplastomic leaves, whereas accumu-
lation in larvae fed with nuclear transgenic leaves was below the limit of reliable detection (fig. SIIC).

CPB resistance of transplastomic potato plants was further assessed by determining the leaf area consumed by CPB larvae and adult beetles. Almost no visible consumption of leaf biomass occurred in St-pdP-ACT leaves (Fig. 2C and fig. S12A), due to complete cessation of larval feed-
ing after 24 hours, even prior to mortality (Fig. 2A). Similarly, adult beetles caused very little additional damage to St-pdP-ACT leaves after 2 days (Fig. 3A). Finally, we exposed whole plants to second-instar larvae (which are generally less sensitive to insecticidal agents than first-instar larvae) and scored survival. This test resulted in 17% survival of the larvae after 5 days of feeding on St-pdP-ACT plants (and 63% survival upon feeding on St-pdP-SHR plants after 6 days; Figs. 3B, fig. S12, B to D, and fig. S13), presumably due to the initial larval growth and development on wild-type leaves. However, the larvae grew very poorly after transfer to the transplastomic plants, and the damage they caused to the leaves was very small (fig. S12, B to D). In nature, CPB larvae typically hatch and feed on the same plant, and therefore they would not enjoy a wild-type diet before feeding on the transplastomic plant.

To ultimately confirm that the plastid-expressed ACT dsRNA silences the actin gene in CPB larvae, we examined actin filaments in the larval midgut, hindgut, and Malpighian tubules by staining with fluorescein isothiocyanate (FITC)–labeled phalloidin. Already after 1 to 2 days of feeding on transplastomic leaves, the larvae displayed disorganized actin filaments, which were particu-
larly obvious in the columnar cells of the midgut (fig. S14). Also, the intensity of phalloidin–FITC labeling progressively decreased with the time of feeding (fig. S14), which suggests that actin de-
ficiency is the cause of death. RNA-dependent RNA polymerase (RdRP) genes are absent from the ge-
omes of insects (18). Therefore, silencing signals activating dsRNAs can be chosen from a vast

REFERENCES AND NOTES

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SUPPLEMENTARY MATERIALS
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Table S1
References (24–37)

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