Length-dependent accumulation of double-stranded RNAs in plastids affects RNA interference efficiency in the Colorado potato beetle

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Abstract

Transplastomic potato plants expressing double-stranded RNA (dsRNA) targeted against essential genes of the Colorado potato beetle (CPB) can be lethal to larvae by triggering an RNA interference (RNAi) response. High accumulation levels of dsRNAs in plastids are crucial to confer an efficient RNAi response in the insects. However, whether length and sequence of the dsRNA determine the efficacy of RNAi and/or influence the level of dsRNA accumulation in plastids is not known. We compared the RNAi efficacy of different lengths of dsRNA targeted against the CPB \( \beta \)-Actin gene (\( \text{ACT} \)) by feeding \emph{in vitro}-synthesized dsRNAs to larvae. We showed that, while the 60 bp dsRNA induced only a relatively low RNAi response in CPB, dsRNAs of 200 bp and longer caused high mortality and similar larval growth retardation. When the dsRNAs were expressed from the plastid (chloroplast) genome of potato plants, we found that their accumulation were negatively correlated with length. The level of dsRNA accumulation was positively associated with the observed mortality, suppression of larval growth, and suppression of target gene expression. Importantly, transplastomic potato plants expressing the 200 bp dsRNA were better protected from CPB than plants expressing the 297 bp dsRNA, the best-performing line in our previous study. Our results suggest that the length of dsRNAs is an important factor that influences their accumulation in plastids and thus determines the strength of the insecticidal RNAi effect. Our findings will aid the design of optimized dsRNA expression constructs for plant protection by plastid-mediated RNAi.

Keywords: Colorado potato beetle, double-stranded RNA, pest control, plastid engineering, RNA interference, transplastomic plants.

Introduction

RNA interference (RNAi) is a post-transcriptional gene silencing mechanism triggered by double-stranded RNA (dsRNA) in eukaryotes. dsRNAs are cleaved by endoribonucleases of the Dicer family into 21–23 bp small interfering RNAs (siRNAs), which then are loaded into the RNA-induced silencing complex (RISC) to cleave and degrade cognate miRNAs (Gordon and Waterhouse, 2007). Since RNAi was first discovered in the model nematode \emph{Caenorhabditis elegans} (Fire et al., 1998),
RNAs has been demonstrated to provide a versatile tool in reverse genetics by exploring gene functions through suppression of gene expression (Wheeler et al., 2003; Tomoyasu and Denell, 2004). In addition, RNAi can be used to modify interactions between organisms, in that dsRNAs produced by one organism can be taken up by another organism and silence genes in that organism, a phenomenon dubbed environmental RNAi or trans‐kingdom RNAi (Cai et al., 2018). For more than a decade, substantial efforts have been made to develop RNAi‐based technologies as a novel approach for insect pest control (Zhang et al., 2017). Efficient delivery of insecticidal dsRNAs (targeting essential insect genes) is the key process in RNAi‐based pest control. Injection of dsRNA into insects is suitable for laboratory research, but not for practical application towards pest control in the field. Therefore, strategies for oral delivery of dsRNA molecules have been explored in both the laboratory and the field, including the use of nanocarriers, detergents, polymers, engineered bacteria and yeasts, and transgenic plants (Zhu et al., 2011; He et al., 2013; Zhang et al., 2015; Murphy et al., 2016; Zheng et al., 2019). Since the costs of kilogram amounts of chemically synthesized dsRNA are very high and large‐scale application to plants in the field is burdened with technical problems, expression of dsRNA in planta represents the method of choice for the control of herbivorous pest insects. Encouragingly, the first plant‐incorporated protectant (PIP) based on RNAi technology (targeting the western corn rootworm, Diabrotica virgifera virgifera) was approved by the US Environmental Protection Agency (EPA) in 2017 and may enter the market soon (Zotti et al., 2018; Schiemann et al., 2019).

Although nuclear‐transgenic plants expressing dsRNAs targeted against essential insect genes can reduce growth and fitness of pest insects, full protection of the plants and efficient killing of the insects have not been achieved (Baum et al., 2007; Mao et al., 2007). The reason is that dsRNAs expressed from the nuclear genome are targets of the plant’s own RNAi machinery and efficiently processed into siRNAs by the Dicer endoribonucleases encoded in the plant genome (Gordon and Waterhouse, 2007). It has been demonstrated that long dsRNAs are much more effective triggers of gene silencing by environmental RNAi in insects than siRNAs. Feeding studies have revealed that dsRNAs of at least 60 base pairs (bp) in length are necessary for an efficient RNAi response in Diabrotica virgifera (Bolognesi et al., 2012) and Tribolium castaneum (Wang et al., 2019). Moreover, since there are no RNA‐dependent RNA polymerases in insects, the silencing signals cannot be amplified in insect cells (Price and Gatehouse, 2008). Thus, the development of a strong RNAi response in the insect critically depends on the continuous uptake of sufficient amounts of exogenously supplied dsRNA. These limitations explain, at least in part, the limited insect resistance conferred by dsRNA expression in nuclear‐transgenic plants.

We recently showed that long dsRNAs can stably accumulate in plastids, a cellular compartment that is derived from former free‐living cyanobacteria, which lack an RNAi pathway (Zhang et al., 2015). Transplastomic potato plants expressing dsRNA targeted against the β‐Actin (ACT) gene of the Colorado potato beetle (CPB, Leptinotarsa decemlineata) were efficiently protected from insect damage and induced a much stronger RNAi response in the beetle than nuclear‐transgenic plants expressing a similar dsRNA construct (Zhang et al., 2015). This was because long ACT dsRNAs (i) accumulate to high levels in plastids owing to the high polyplody of the plastid genome with up to 10 000 identical copies per cell, and (ii) remained intact due to the absence of an RNAi pathway (and the absence of Dicer‐like dsRNA‐specific endoribonucleases) from plastids. Although the same promoter (the plastid 16S ribosomal RNA gene promoter, Prrn) was used to drive transcription of three different dsRNA constructs in plastids, very different dsRNA accumulation levels were obtained: ~0.4% of the total cellular RNA for ACT, ~0.05% for Shrub (SHR), and ~0.1% for ACT+SHR (fusion of ACT and SHR). The factors that determine the efficiency of target gene silencing in CPB and the dsRNA accumulation levels in plastids are currently unknown (see Supplementary Table S1 at JXB online).

In the present study, we set out to evaluate the effects of dsRNA length on the efficiency of RNAi as evidenced by the induction of gene silencing and mortality in CPB. We also compared the accumulation levels of dsRNAs of different lengths in transplastomic plants. Our results show that, while all dsRNAs of 200 bp and longer had similar effects on the induction of gene silencing, the dsRNA accumulation levels in plastids were negatively correlated with the length of the dsRNAs. We also established a positive correlation between dsRNA accumulation levels in plastids and RNAi efficacy in CPB.

Materials and methods

Insect and plant material and growth conditions

CPB (L. decemlineata) larvae and beetles were collected from a potato field in Urumqi (43.82°N, 87.61°E), Xinjiang Uygur Autonomous Region, China. Insects were reared in an insectary at 28±1 °C under a 14 h light–10 h dark photoperiod and 50–60% relative humidity using leaves from wild‐type potato plants as feed. Adult CPBs were allowed to lay eggs, which were collected and transferred onto fresh wild‐type potato leaves.

Potato (Solanum tuberosum cv. Désirée) plants for plastid transformation experiments were grown under aseptic conditions on MS medium supplemented with 30 g l−1 sucrose (pH adjusted to 5.7) (Murashige and Skoog, 1962) and solidified with 0.6% Micro agar (Duchefa). Transplastomic lines were rooted in the same medium and grown to maturity in soil under standard greenhouse conditions.

In vitro RNA synthesis

dsRNA was synthesized in vitro using the T7 RiboMAX™ Express RNAi System (Promega, cat. no. P1700, USA) according to the manufacturer’s instructions. ACT fragments of different length were PCR amplified using specific primers containing the T7 promoter sequence (see Supplementary Table S2). The PCR products were purified with a gel extraction kit (Omega, China). The reaction mixture consisted of 2 µl T7 Express Enzyme Mix, 10 µl RiboMAX™ Express T7 2 × Buffer, and 1 µg of DNA template. The mixture was incubated at 37 °C for 2–6 h and 70 °C for 10 min, then slowly cooled to room temperature (~20 min) to anneal of the dsRNA. The DNA template and single‐stranded RNA (ssRNA) were removed by DNase (removing DNA template) and RNase A (removing ssRNA) treatments, respectively. The yield of dsRNA was determined by ultraviolet absorbance at a wavelength of 260 nm with a Nano Photometer (Implen, Germany) and the integrity of the full length was verified by RNA gel electrophoresis.
For ssRNA synthesis, the ACT200 fragment was amplified with primer pair act200-F/T7act2000Rev (see Supplementary Table S2). In vitro transcription reactions were conducted with T7 RNA polymerase (Thermo Fisher Scientific, USA) following the manufacturer's instructions. The RNA yield was determined with a Nano Photometer (Implen, Germany) and kept at −80°C until further use.

**Insect bioassays**

dsRNA feeding bioassays were performed under controlled condition as described previously (Zhu et al., 2011). Third-instar CPB larvae were starved for 24 h prior to initiation of feeding assays. Equal amounts (16 ng, in 50 µl ddH2O) of dsRNAs of different lengths were painted onto fresh potato leaves covering identical areas of 2×2 cm, thus adjusting the concentration of dsRNA to 4 ng cm⁻². ddH₂O-painted leaves were used as control. Sufficient dsRNA-coated fresh leaves were provided to the insects and exchanged every day for the duration of the bioassays to ensure the stability of foliar-applied dsRNA and optimal CPB feeding (San Miguel and Scott, 2016). Three groups of insects per treatment were investigated, and each group comprised 10 larvae. The body weight of larvae was recorded at day 3, 4, and 5 for all groups. The mortality was recorded daily.

For larval performance assays on transplastomic potato lines (n≥6), third-instar CPB larvae were allowed to feed on young leaves detached from 2-month-old transplastomic or wild-type (as control) potato plants. The body weight of the larvae was recorded at the beginning (day 0) of the assay and at day 2, 3, and 4 for all groups. The mortality was recorded daily.

**Quantitative real-time polymerase chain reaction**

Total RNA samples were extracted with the RNAiso plus reagent (Takara, Japan). After digestion with gDNA digester (Yeasen, China), cDNA was synthesized using the RevertAid First Strand cDNA Synthesis Kit (Yeasen). The reference gene RP18 was chosen as internal control (Pfaff et al., 2004; Huggett et al., 2005). The reaction mixture consisted of 5 µl TB Green Premix Ex Taq II (Tli RNaseH Plus), 1 µl of cDNA, and 0.25 µl of forward and reverse primers (see Supplementary Table S2; 10 µM) in a final reaction volume of 10 µl. The qPCR protocol included an initial denaturation step at 95°C for 2 min, followed by 40 cycles of 95°C for 3 s, 60°C for 30 s and 72°C for 30 s. The relative expression of β-Actin was calculated by the 2⁻ΔΔCT method (Livak and Schmittgen, 2001; Shi et al., 2013). All experiments were repeated in triplicate.

**Vector construction**

The plastid transformation vectors used in this study were constructed based on the previously described vector pYY12 (Wu et al., 2017). The gfp cassette in pYY12 was replaced by the dsRNA expression cassette excised from pZJ197 (Zhang et al., 2015) by digestion with HincII and NolI, resulting in plasmid pWW1. ACT fragments were obtained by PCR amplification with primer pairs ACT700-F/AcT700-R, ACT400-F/AcT400-R, and ACT200-F/AcT200-R (see Supplementary Table S2), using a clone containing the full-length β-Actin cDNA as template. The resulting PCR products were digested with SflI and SadI, and cloned into the similarly cut vector pWW1, generating plastid transformation vectors pWW3, pWW4, and pWW7, respectively.

**Potato plastid transformation**

Potato plastid transformation was performed as described previously (Zhang et al., 2017). Briefly, young leaves from aseptically grown potato plant were bombarded with DNA-coated 0.6 µm gold particles using a BDS-1000/He Biolistic Particle Delivery System (Bio-Rad, Hercules, CA, USA). Plasmid DNA for plastid transformation was prepared by using the Nucleobond Xtra Plasmid Midi Kit (Macherey-Nagel, Germany). Spontaneous antibiotic-resistant mutants were eliminated by screening on shoot regeneration medium supplemented with both spectinomycin and streptomycin (Bock, 2001). Transplastomic events were confirmed by Southern blot analyses and subsequently purified to homoplasy.

**Isolation of nucleic acids and gel blot analysis**

Total DNA from wild-type and transplastomic plants was extracted by a cetyltrimethylammonium bromide (CTAB)-based method (2% CTAB, 1.4 mM NaCl, 0.1 mM Tris–HCl pH 8.0, 20 mM EDTA pH 8.0; Doyle and Doyle, 1990). Total RNA was isolated using the RNAiso plus reagent following the manufacturer's instruction (Takara, Japan). For Southern blot analyses, samples of 5 µg total DNA were digested with the restriction enzymes Agel and Mbol for 12–16 h, separated by electrophoresis in 1% agarose gels, and transferred onto Hybond nylon membranes (GE Healthcare) by capillary blotting. A PCR product covering a portion of the psbZ coding region (You et al., 2019) was used as a hybridization probe.

For RNA gel blot analyses, RNA samples were denatured, separated in formaldehyde-containing 1% agarose gels and blotted onto Hybond nylon membranes (GE Healthcare). A PCR product generated by amplification of ACT cDNA with primer pair act-N-F/act-N-R served as probe to determine dsRNA amounts. The expression of potato plastid-encoded psbA gene served as internal control. The psbA gene-specific probe was synthesized using primer pair psbA-N-F/psbA-N-R (see Supplementary Table S2). The probe was labeled with the DIG-High Prime DNA Labeling and Detection Starter Kit II following the manufacturer's instructions (Roche, USA).

**Statistical analysis**

Survival curves were analysed using the Kaplan–Meier method. The log-rank test was used to evaluate the significance of differences between two groups. Quantitative real-time polymerase chain reaction (qRT-PCR) data were used to calculate the relative expression of β-Actin by the 2⁻ΔΔCT method. Data for qRT-PCR and body weight were analysed with one-way ANOVA coupled with Bonferroni (equal variances) or Dunnett’s T3 (unequal variances) correction multiple comparison test. A value of P<0.05 was considered significantly different. Data were statistically analysed with SPSS version 19.0. Figures were drawn with GraphPad Prism 7 and SnapGene 3.2.1.

**Results**

**Effect of dsRNAs of different lengths on RNAi efficacy in Colorado potato beetle larvae**

To avoid potential effects from different GC contents of the selected dsRNA target regions, we cloned β-Actin fragments (ACTs) of different lengths (60, 200, 297, 400, and 700 bp) but similar GC content (~52.2%). The cloned gene fragments were then used as templates for dsRNA synthesis by in vitro transcription (see Supplementary Fig. S1). To assess the effects of dsACT length on RNAi efficacy in CPB larvae, dsACT-painted leaves with defined amounts of dsRNA (4 ng cm⁻²) were fed to third-instar larvae. Compared with the control (fed with H₂O-painted leaves), feeding of larvae with dsACT of lengths ranging from 200 to 700 bp resulted in significantly increased mortality, inhibited growth, and suppressed β-Actin expression (Fig. 1). No significant differences in killing efficiency, weight gain of survivors, and level of gene silencing were detected between dsACTs of lengths between 200 and 700 bp. By contrast, dsACT60 was much less effective than dsACTs of 200 bp and greater length. Compared with the control, only slightly retarded larval growth was observed (Fig. 1C), but no significantly increased mortality and no significant target gene silencing. In summary, the RNAi effects obtained with dsACTs of different length were shown to be 200 bp=297 bp=400 bp>700 bp=600 bp.
Generation and molecular characterization of transplastomic potato plants expressing dsACTs

Having seen very similar RNAi effects of dsACTs above a length of 200 bp on CPB, we next constructed potato plastid transformation vectors for expression of dsACT200 (vector pWW7), dsACT400 (vector pWW4) and dsACT700 (vector pWW3) (Fig. 2A). The constructs were introduced into the potato plastid genome by particle gun-mediated (biolistic) chloroplast transformation. The expression of dsACTs in plastids is driven by two convergent copies of the tobacco plastid ribosomal RNA operon promoter (Prrn). The dsRNA could be formed by annealing two complementary RNA transcripts in plastids. The expression of dsACTs in plastids is driven by two convergent copies of the tobacco plastid ribosomal RNA operon promoter (Prrn). The dsRNA could be formed by annealing two complementary RNA transcripts in plastids. The plastid-specific selectable marker gene aadA (conferring spectinomycin resistance) is driven by the chloroplast psbA promoter and the 3′-untranslated region of the rbcL gene from the unicellular green alga Chlamydomonas reinhardtii. Selection for spectinomycin resistance produced several independent transplastomic potato lines for each construct, and two independent transplastomic lines per construct were selected for further characterization. Transgene integration into the plastid genome by homologous recombination was confirmed by Southern blot analysis. The homoplasmic status of the transplastomic potato lines (i.e. the absence of residual wild-type copies of the highly polyploid plastid genome) was evidenced by the absence of a hybridization signal for the 2.13 kb fragment diagnostic of the wild-type plants (St-wt) and exclusive presence of the 2.51 kb fragment expected in transplastomic lines (Fig. 2B).

Determination of dsRNA accumulation levels in plastids

Having obtained the transplastomic potato plants, we next examined the accumulation levels of the dsACTs of different lengths in plastids by northern blot analysis. For comparison, we included the previously generated transplastomic potato line St-ptDP-ACT21 (expressing dsACT297; referred to as St-ACT297; Zhang et al., 2015). The data revealed that the accumulation levels of dsACTs in the different transplastomic lines were St-ACT700<St-ACT400<St-ACT297<St-ACT200. These findings indicate a length-dependent mode of dsRNA accumulation in plastids in that the accumulation levels decrease with the length of the dsRNA (Fig. 2C). However, there were no differences in expression of plastid-encoded psbA gene in different transplastomic lines (see Supplementary Fig. S2).
excluding the possibility that different \textit{dsACT} accumulation levels were due to RNA degradation during extraction. When compared with a dilution series of \textit{in vitro} synthesized RNA, \textit{dsACT} accumulation levels in St-\textit{ACT}200 line were estimated to be \( \sim 0.6\% \) of the total cellular RNA (Fig. 2D), which is \( \sim 1.5 \) times as high as in St-\textit{ACT}297 (\( \sim 0.4\% \) of the total cellular RNA) (Fig. 2E).

We also noted evidence of substantial dsRNA degradation in the transplastomic lines expressing longer \textit{dsACT} fragments (Fig. 2C), suggesting that the lower levels of \textit{dsACT} accumulation in these plants are caused by RNA instability. This interpretation is also consistent with all constructs being driven by identical promoters, thus likely resulting in identical rates of transcription.

\section*{RNAi effects of transplastomic plants on the Colorado potato beetle}

We next evaluated whether there were differences in the level of insect resistance conferred by the transplastomic potato lines expressing different lengths of \textit{dsACT}. Third-instar CPB larvae were chosen for the insecticidal bioassays, because they display relatively high survivorship compared with first- or second-instar CPB larvae when exposed to lethal dsRNAs (Guo \textit{et al.}, 2015).

Our bioassays revealed that, compared with the wild-type control (St-wt), feeding of CPB with leaves from St-\textit{ACT}200, St-\textit{ACT}297, and St-\textit{ACT}400 lines resulted in high mortality of the larvae. The CPB larvae fed with these lines showed similarly strong
reductions in weight gain (Fig. 3C). Interestingly, starting from day 2, the larvae almost completely ceased feeding on the St-ACT200, St-ACT297, and St-ACT400 plants (see Supplementary Fig. S3). By contrast, no significant mortality was observed in CPB larvae fed on the St-ACT700 line that showed the lowest dsACT accumulation levels (Figs 2C, 3A). Although resulting in reduced larval weight gain and detectable induction of gene silencing in CPB larvae compared with the control (feeding on wild-type plants), the St-ACT700 line causes a much weaker RNAi effect on the CPB larvae than does the other St-Act lines (Fig. 3B,C). The RNAi effects on CPB larvae fed with St-Act transplastomic plants were St-ACT700<St-ACT400<St-ACT297<St-ACT200, and thus are strikingly correlated with the dsACT accumulation levels in plastids (Fig. 2C). The St-ACT200 plants caused even higher rates of killing of CPB larvae than the St-ACT297 plants, the best-performing transplastomic potato plants reported previously (Zhang et al., 2015) (Fig. 3A; Supplementary Fig. S2).

Discussion
RNAi has emerged as a promising strategy for insect pest control. However, the factors influencing the efficiency of environmental RNAi in insects are not fully understood. The length of the dsRNA trigger has been demonstrated to be an important factor that affects RNAi efficacy in T. castaneum (Miller et al., 2012; Wang et al., 2019) and D. virgifera (Bolognesi et al., 2012). It was reported that dsRNAs of at least 60 bp in length are required for efficient RNAi. siRNAs of 21 nt, the products of dsRNA processing by Dicer, had little effect on D. virgifera. Similarly, dsRNAs of 69 bp, but not of 30 bp, were reported to induce the RNAi responses in T. castaneum (Miller et al., 2012). A number of factors, including the cellular uptake efficiency of dsRNAs and dsRNA degradation in the midgut of insects, have been implicated in the length-dependent effects of dsRNA. Based on the established threshold length of ~60 bp, we tested dsRNAs of lengths ≥60 bp that target the CPB β-Actin gene (dsACTs). Consistent with previous studies, we found that dsACTs of 200 bp and longer are much more potent than dsRNA of 60 bp. Remarkably, no significant increase in RNAi efficiency was observed with dsRNAs of lengths greater than 200 bp (Fig. 1). The finding that expression of very long dsRNAs does not improve the insecticidal activity is important and should be considered in future efforts to design optimized RNAi constructs for pest control.

Fig. 3. Feeding assays of CPB larvae on transplastomic potato plants. (A) Kaplan–Meier survival curves of third-instar CPB larvae upon feeding on detached leaves of St-Act lines. The log-rank test was used to assess the significance of differences between two survival curves. ***P<0.001, *P<0.05; NS, not significant. (B) Relative expression levels of β-Actin in CPB larvae at day 3 of the assay. Gene expression levels were set as 1 in CPB larvae fed with wild-type (St-wt) potato plants. Data are presented as means ±SD (n=5). (C) Mean weight of CPB larvae at the indicated days of feeding. Data are presented as means ±SE (n=30). The letters above each bar in (B) and (C) indicate the significance of differences as determined by one-way ANOVA in SPSS (Bonferroni’s test, P<0.05). (This figure is available in color at JXB online.)
We previously showed that plastids can be engineered to produce dsRNA to control CPB. We also reported that different dsRNAs can accumulate to different levels in chloroplasts (Zhang et al., 2015). Plastid genes are transcribed by two different RNA polymerases in angiosperms: a phage-type nucleus-encoded RNA polymerase (NEP), and a eubacterial-type plastid-encoded RNA polymerase (PEP). Two RNA polymerases can jointly or individually transcribe different plastid genes (Hajdukiewicz et al., 1997; Börner et al., 2015). The Prrn promoter used to drive dsRNA expression in this study is mainly transcribed by PEP (Suzuki et al., 2003). Our results indicate that dsRNA accumulation levels decrease with increasing dsRNA length (Fig. 2C). As the same expression signals (Prrn) were used to produce dsACTs in all of our transplastomic lines, it seems reasonable to assume that the different (length-dependent) accumulation levels of dsACTs are not due to different rates of transcription, but rather have a post-transcriptional cause. Possible reasons for the length-dependent accumulation of dsRNAs could be related to the efficiency of dsRNA formation (by annealing of the two complementary single strands) and/or decreasing dsRNA stability with length. So far, no dsRNA processing or degrading enzymes have been found in plastids (Stern et al., 2010). We, therefore, suspect that the accumulation levels of dsACTs in plastids are more likely to be determined by the efficiency of the dsRNA annealing process. dsRNA formation from single strands is conceivably negatively correlated with length, in that long single-stranded RNAs have a higher propensity to form secondary structures than do short ones, which may impede perfect annealing to dsRNAs. Imperfectly annealed single strands may form loops and bulges that then are attacked by endoribonucleases present in plastids.

However, our data reported here do not fully explain the previous observation that three types of dsRNAs (dsACT, dsSHR, and dsACT+SHR) accumulated to very different levels in plastids (Zhang et al., 2015) (see Supplementary Table S1). For example, dsSHR (220 bp in length) was shorter than dsACT (297 bp in length), while the accumulation level of dsSHR in plastids was at least 8-fold lower than that of dsACT. This suggests that dsRNA length is not the only factor determining the accumulation levels of dsRNAs in plastids. Whether other factors like primary sequence and GC content of dsRNAs influence dsRNA accumulation in plastids remains to be investigated. Conceivably, these factors could also affect the efficiency of strand annealing and, in this way, act similarly as discussed above for RNA length.

Since insects have no RNA-dependent RNA polymerase, the RNAi efficiency in insects is believed to be largely dependent on the amount of environmental dsRNAs that can be delivered to and taken up by insect cells (Price and Gatehouse, 2008). This assumption is consistent with our results that the RNAi effects of St-ACT plants on CPB were strongly positively correlated with dsACT accumulation levels in plastids (Figs 2C, 3). dsACT200 was found to accumulate to the highest level (~0.6% of total cellular RNA in St-ACT200 lines; Fig. 2C, D) and resulted in one of the strongest RNAi effects in CPB larvae (Zhang et al., 2015). Thus, shortening the length of dsRNAs expressed in transplastomic plants may be a useful strategy to increase dsRNA accumulation levels in plastids and, in this way, enhance their insecticidal effect. It is worth noting that, although the plastid-mediated RNAi approach overcomes the main drawbacks related to dsRNA expression from the plant’s nuclear genome for pest control (Zhang et al., 2017), biosafety issues and regulations on GM-based products have to be addressed before the practical application of this technology. Widespread use of RNAi in insect pest management can also be restricted by variable RNAi efficiency among insects, and the potential development of resistance in insect populations (Zhu and Pali, 2020).

Taken together, our data revealed that dsRNA length is an important factor that determined dsRNA accumulation levels in plastids, and influences the efficacy of the RNAi response triggered by transplastomic plants in pest insects. This study will facilitate the design of optimized expression constructs to maximize dsRNA accumulation levels in plastids and increase the efficacy of plastid-mediated RNAi for pest control.

Supplementary data
Supplementary data are available at JXB online.

Fig. S1. In vitro synthesis of dsACTs of different lengths.

Fig. S2. Analysis of psbA transcript accumulation by northern blotting.

Fig. S3. Leaves of wide-type potato plants and transplastomic plants expressing different length of dsACTs consumed by third-instar CPB larvae.

Table S1. Summary of dsRNA accumulation levels in transplastomic lines expressing different dsRNA constructs (Zhang et al., 2015).

Table S2. List of oligonucleotides used in this study.

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Author contributions
JZ conceived the project; WH and WX performed the research; LX and JZ analysed the data. KF and WG supported the research and provided the CPB resources. RB and JZ wrote the article with contributions of all other authors.

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