The initiation of RNA interference (RNAi) by topically applied small interfering RNA has potential applications for plant functional genomics, crop improvement and crop protection, but the primary obstacle for the development of this technology is the efficient delivery of RNAi effectors into the cell. The plant cell wall is a particularly challenging barrier for the delivery of macromolecules because many of the transfection agents that are commonly used with animal cells produce nanocomplexes that are significantly larger than the size exclusion limit of the cell wall. Here, we illustrate the use of a class of very small nanoparticles, called carbon dots, for delivering small interfering RNA into the model plants Nicotiana benthamiana and tomato (Solanum lycopersicum). Low-pressure spray application of these formulations with a spreading surfactant resulted in strong silencing of GFP transgenes in both species. The delivery efficacy of carbon dot formulations was also demonstrated by the silencing of endogenous genes that encode two subunits of magnesium chelatase, an enzyme necessary for chlorophyll synthesis. The strong visible phenotypes observed with the carbon dot–facilitated delivery were validated by measuring significant reductions in the target gene transcript and/or protein levels. Methods for the delivery of RNAi effectors into plants, such as the carbon dot formulations described here, could become valuable tools for gene silencing in plants with practical applications in plant functional genomics and agriculture.

RNA interference (RNAi) comprises interrelated pathways that mediate transcriptional gene silencing by methylation of genomic sequences, posttranscriptional gene silencing by cleavage of targeted RNA sequences, or translational repression by binding to targeted transcripts (Matzke and Matzke, 2004; Frizzì and Huang, 2010). In plants, these pathways have a role in resistance to pathogens (Rosa et al., 2018) and are also required for normal development (Liu et al., 2017). The use of RNAi to silence specific genes has become a valuable tool for plant functional genomics.
that enhance delivery into animal cells have been described (Kozielski et al., 2013). Common classes of these transfection agents include lipid nanoparticles, cationic polymers, cell-penetrating peptides, and inorganic nanoparticles. The nanocomplexes that are formed by the interaction of these transfection agents with nucleic acids provide some protection from nucleases and facilitate cellular uptake by endocytosis or membrane fusion. There have been reports describing the use of transfection agents for delivery into plant cells (Unnamalai et al., 2004; Cheon et al., 2009; Eggenberger et al., 2011; Lakshmanan et al., 2013; Numata et al., 2014; Ziemienowicz et al., 2015; Cheon et al., 2017; Kimura et al., 2017; Miyamoto et al., 2019). The efficiency of many transfection agents, however, may be limited by delivery barriers that are unique to plants (Fig. 1). The plant cell wall is a particularly challenging barrier for the delivery of RNA or other macromolecules. The dense polysaccharide matrix of the cell wall has a size exclusion limit that is between 3 and 10 nm in diameter (Carpita et al., 1979; Baron-Epel et al., 1988; Carpita and Gibeaut, 1993). Many of the nanocomplexes used to transfact nucleic acids have a size in the range of 100 to 200 nm, which is 10- to 20-fold larger than the size exclusion of the plant cell wall. There have been several reports describing the use of smaller nanostructures, such as single-walled carbon nanotubes (Golestanipour et al., 2018; Demirer et al., 2019; Kwak et al., 2019) and DNA nanoparticles (Zhang et al., 2019), for the delivery of nucleic acids into plant cells. Delivery of small interfering RNA (siRNA) and transient silencing of a GFP transgene have been achieved by infiltration of formulations into leaf tissues with a needleless syringe (Zhang et al., 2019). The use of clay nanosheets to stabilize longer dsRNA and provide protection from virus infection in plants has also been reported (Mitter et al., 2017).

There are additional classes of nanomaterials that may be well suited for plant delivery. Quantum dots, for example, are small nanoparticles that have shown good efficacy in the transfection of animal cells (Yezhelyev et al., 2008). A significant disadvantage to quantum dots is that they are most often made from heavy metals. In recent years, carbon dots have received a considerable amount of attention as a “green” alternative to quantum dots (Reckmeier et al., 2016; Yao et al., 2019). Much of the interest in carbon dots has evolved around their optical properties, which have practical applications for bioimaging, photocatalysis, photovoltaic cells, and light-emitting diodes. The uptake of carbon dots in plants has been studied (Li et al., 2016, 2018; Qian et al., 2018), but their use for delivery of nucleic acids into plant cells has not yet been reported. There are reports describing the use of carbon dots for transfection of plasmids, long dsRNA, and siRNA into animal cells (Liu et al., 2012; Wang et al., 2014; Das et al., 2015; Pierrat et al., 2015). With a high ratio of carbon dots to nucleic acids, relatively small nanocomplexes can be formed. Using carbon dots with an average size of 19.7 nm, a nanocomplex with plasmid

![Figure 1. Strategy for delivery of siRNA into plants. The first barrier for delivery is the cuticle, a water-impermeable layer that covers all the above-ground parts of the plant. Stomates, the pores that allow for gas exchange, can be a point of entry for formulations. With a spreading surfactant, formulations can flow across the leaf surface and “flood” stomates for delivery to the mesophyll cells (Schönherr and Bukovac, 1972). Once in the leaf apoplast, nanocomplexes containing the siRNA cargo need to diffuse through the cell wall to reach the plasma membrane. The relatively small size-exclusion limit of the cell wall (<10 nm) would likely restrict the movement of larger nanocomplexes. Smaller nanocomplexes can reach the plasma membrane and facilitate cellular uptake by endocytosis. Escape from the endomembrane vesicles can be achieved by a phenomenon called the “proton-sponge” effect, which causes osmotic swelling and lysis of the vesicles (Behr, 1997). Some portion of the siRNA may be released from the nanoparticles by changes in pH between cellular compartments or by the competitive binding of naturally occurring biomolecules.](https://academic.oup.com/plphys/article/184/2/647/6117875)
DNA was only marginally larger at 20.6 nm when the mass ratio of carbon dots to DNA was 32 (Pierrat et al., 2015). Wang et al. showed that carbon dots with an average size of \( \approx 3.5 \text{ nm} \) formed nanocomplexes with siRNA that were \( \approx 5 \text{ nm} \) in size. Because of their small size, we hypothesized that carbon dots might be useful for the delivery of siRNA through the plant cell wall and subsequent barriers.

RESULTS

Preparation and Purification of Carbon Dots

Carbon dots can be synthesized by a “top-down” approach, which involves the decomposition of structured carbon precursors such as graphene. Alternatively, the “bottom-up” approach begins with the carbonization of simple precursors, such as organic acids, sugars, or amino acids (Yao et al., 2019). The bottom-up methods for producing carbon dots usually involve a hydrothermal reaction or pyrolysis of the carbon precursor to produce nanoparticles with sizes that are typically between 1 and 10 nm. Surface functionalization/passivation of the carbon dots can increase the colloidal stability and allow for the binding of various ligands. For example, functionalization with amines produces particles with a positive charge that can bind with the negative charges of the phosphate backbone in nucleic acids. Polyethyleneimine (PEI) is a commonly used cationic polymer for the functionalization of carbon dots. Citrate-derived carbon dots functionalized with branched PEIs (bPEIs) have been used to deliver plasmid DNA and siRNA to animal cells (Liu et al., 2012; Pierrat et al., 2015).

Functionalization of carbon dots with PEI is often done in a “one-pot” synthesis where carbonization of the precursor and functionalization occur at the same time. This is possible because aqueous solutions of PEI are relatively thermostable. There are several reports describing the synthesis of carbon dots directly from PEI or other amines with the assistance of \( \text{H}_2\text{O}_2 \) (Zhou et al., 2015) or by performing the reactions in chloroform (van Dam et al., 2017). In those examples, PEI serves as both the carbon source for the core and the nitrogen source for surface passivation. In the work described here, carbon dots were produced directly from bPEIs of various \( M_r \) by heating solutions in a mixture of chloroform and methanol to 155°C for a relatively short time. The bPEI-derived carbon dots had...
an absorption maximum of 363 nm and produced a blue fluorescence with an emission maximum at 460 nm (Supplemental Fig. S1), characteristic of nitrogen-doped carbon dots.

The carbon dot preparations likely contained a heterogeneous mixture of precursors, by-products, and carbon dots of different sizes and physical properties. Therefore, the preparations were fractionated by size-exclusion chromatography (Fig. 2A), and the size distributions within the fractions was determined by dynamic light scattering (Fig. 2B). Based on the size exclusion chromatography and dynamic light scattering measurements, the size distribution of the carbon dots associated well with the $M_r$ of the PEI precursors. The smallest PEIs used in this study, with average $M_r$ values of 1,200 and 1,800 D, produced the smallest carbon dots. The carbon dots produced from 5-, 10-, or 25-kD bPEIs were progressively larger.

Gel retardation assays were used to demonstrate the binding of dsRNA to carbon dots and enhanced resistance of the bound dsRNA to nuclease degradation (Supplemental Fig. S2). The *Escherichia coli* RNase III and a 124 bp dsRNA were used for these in vitro protection assays because this enzyme has a very high level of activity with longer dsRNA. In assays that contained the dsRNA alone, significant degradation was observed after 1 min and the dsRNA was mostly degraded by 15 min. When dsRNA was formulated with the carbon dots, there was little apparent degradation after a 60-min incubation with RNase III. This enhanced resistance to nuclease could be significant for efficacy in plants, which contain high levels of RNase activity in the extracellular apoplast (Sangaev et al., 2011).

**Carbon Dot Formulations Are Efficacious in Silencing GFP in the 16C Line of Nicotiana benthamiana**

The GFP-expressing 16C line of *N. benthamiana* is an often-used model for the study of RNAi (Ruiz et al., 1998; Dalakouras et al., 2016; Bally et al., 2018). In wild-type plants, chlorophyll in the leaves displays a strong red fluorescence under UV or strong blue lights. In the GFP transgenic lines, this red fluorescence is masked by the green fluorescence. The silencing of GFP is then easily detected by the unmasking of the red chlorophyll fluorescence. For the silencing experiments in this study, 22-mer siRNAs were used. Several lines of research have demonstrated that the initiation of silencing with 22-mer siRNAs may result in more secondary siRNA production, which can enhance RNAi phenotypes (Mlotshwa et al., 2008; Manavella et al., 2012; Dalakouras et al., 2016; Taochy et al., 2017). The sequences of the GFP-targeting 22-mer and other siRNAs used in this study are shown in Supplemental Table S1.

![Image](https://academic.oup.com/plphys/article/184/2/647/6117875)

**Table 1. Percent silenced area with different fractions of CD-5K**

Different fractions of the CD-5K preparation were formulated with the GFP-targeting 22-mer siRNA at a concentration of 8 ng μL<sup>-1</sup>. The mean percent silenced area relative to the entire leaf area was calculated digitally with four replicates per treatment. Means were separated using a Student’s t test. The fractions not connected by a letter are statistically different ($\alpha = 0.05$). Images used for the analysis are included in Supplemental Fig. S5.

| Fraction | Connected Letters | Silenced Area % | SE |
|----------|-------------------|-----------------|----|
| Fraction 5 | A — — | 43.6 | 3.81 |
| Fraction 6 | A — — | 36.2 | 6.13 |
| Fraction 7 | A B — | 35.6 | 7.33 |
| Fraction 8 | A B — | 28.5 | 5.59 |
| Fraction 9 | — B — | 20.4 | 2.36 |
| Fraction 10 | — — C | 4.9 | 1.14 |
preparation. Fraction 6 from the 10-kD preparation, for example, displayed much higher silencing efficacy than fraction 4 from the same preparation (Fig. 3). As part of the optimization process, additional fractions from the 5-kD bPEI preparations (CD-5K) were tested for efficacy in silencing GFP in the 16C line. The carbon dots from these different fractions ranged in size between 2.7 and 3.9 nm (Supplemental Table S2). Based on image analysis of the silenced area, fraction 5 from the CD-5K preparation was the most efficacious (Table 1; Supplemental Fig. S5). The later fractions, which contain smaller carbon dots, were less efficacious in silencing GFP.

For molecular validation of gene silencing, the CD-5K fraction 5 was used. The application of a carbon dot formulation with a nontargeting siRNA did not impact the typical green fluorescence of the 16C line under blue lights. Plants that were sprayed with a formulation containing a GFP-targeting 22-mer siRNA displayed a strong red fluorescence that covered most of the leaf (Fig. 4A). The level of GFP transcript reduction, measured by reverse transcription quantitative PCR (RT-qPCR), was 84% (Fig. 4B). Immunoblot analysis showed a similar reduction in GFP protein levels at 87% (Fig. 4, C–E). This level of GFP silencing was enough to initiate the systemic spread of silencing. By 12 d after treatment, GFP silencing in newly emerging leaves became apparent (Supplemental Fig. S6).

Carbon Dot Formulations Are Efficacious in Silencing Endogenous Genes in N. benthamiana

The silencing of endogenous genes was tested by targeting the genes encoding the H and I subunits of magnesium chelatase (CHLH and CHLI), an enzyme necessary for chlorophyll synthesis. True leaves 3 and 4 from 17-d-old N. benthamiana plants were sprayed with carbon dot formulations containing a nontargeting siRNA (Fig. 5A), a CHLI-targeting siRNA (Fig. 5B), or a CHLH-targeting siRNA (Fig. 5C). Leaves that were sprayed with formulations targeting either the H or I subunits displayed spots and patches of bleaching that is indicative of reduced chlorophyll accumulation, whereas leaves that were sprayed with the nontargeting siRNA displayed no visible phenotype. Over several experiments, the bleaching phenotype was strongest on the younger leaf 4 and with the formulation targeting the CHLI gene. Analysis of CHLI transcript levels by RT-qPCR showed a 79% reduction in the phenotypic tissues at 5 d after treatment (Fig. 5D). The bleaching phenotype persisted for the duration of the experiments on the application leaves, which was up to 20 d after treatment (Supplemental Fig. S7).

The CHLH silencing phenotype was used to evaluate the stability of carbon dot formulations. A single batch of formulation with the CHLH-targeting 22-mer siRNA was sprayed on plants 1 d, 1 week, or 2 weeks after preparation. The plants were imaged 5 d after treatment (Supplemental Fig. S8). With the spray application at 1 week, there did not appear to be a significant loss in the efficacy of the carbon dot formulation; indicating that the siRNA is mostly intact, and that the formulation has good colloidal stability. The formulation that was stored for 2 weeks at room temperature displayed a...
bleaching phenotype, but there did appear to be some reduction in the phenotype.

Carbon Dot Formulations Are Efficacious for siRNA Delivery and Silencing in Tomato

The efficacy of carbon dot formulations for siRNA delivery and silencing in tomato (Solanum lycopersicum) was tested with a line expressing enhanced GFP (eGFP). Plants that were treated with a formulation containing a nontargeting siRNA displayed a strong green fluorescence (Fig. 6A). With formulations containing the eGFP-targeting siRNA, silencing was apparent as spots and patches at the lower siRNA concentrations (2 and 4 ng μL⁻¹). At an siRNA concentration of 8 ng μL⁻¹, the silencing phenotype covered most of the leaf. Immunoblot analysis of whole leaf extracts showed an 88% reduction in GFP protein levels in tomato leaves that were treated with the formulation containing 8 ng μL⁻¹ of the GFP-targeting siRNA (Fig. 6, B–D).

The stomatal flooding application method with the spreading surfactant works well with fully expanded leaves. In younger leaves, spray application of carbon dot formulations results in strong silencing toward the tip with limited silencing at the base (Fig. 7). In basipetal plants, such as tomato, functional stomates develop first at the leaf tip. The limited silencing at the base of the young leaves is likely due to reduced flooding where stomates have not yet fully developed. With the limitations in stomatal flooding, other methods may be more amenable for silencing genes in very young tissues. Delivery methods for DNA or RNA that rely on the physical disruption of barriers have also been used (Shang et al., 2007; Dalakouras et al., 2016). These physical delivery methods appear to be most effective with young tissues and could be complementary to the carbon dot-mediated delivery described here.

An Alternative Method to Produce Carbon Dots

Given the high level of activity observed with CD-5K, a method was developed to produce carbon dots from Glc with 5 kD bPEI for functionalization in a “one-pot” synthesis. These carbon dots, which were produced at a lower temperature and in an aqueous solution, showed high levels of activity for the silencing of GFP in tomato (Fig. 8A) and of GFP and CHLH in N. benthamiana (Fig. 8, B and C). With the lower temperature and the aqueous solvent used for production of the Glc carbon dots, methods could be developed that would require less specialized lab equipment.

DISCUSSION

Transient gene silencing methods, such as VIGS and A. tumefaciens-mediated infiltration, have been important tools for studying the function of plant genes. The development of delivery methods for topically applied siRNA would be another valuable tool for plant functional genomics. In addition to its simplicity, a topical RNAi delivery method would not require the containment of treated plants, making this method better suited for field studies. As discussed in the introduction, the reagents that are most often used to transfected nucleic acids into animal cells form nanocomplexes that

Figure 5. Silencing of the magnesium chelatase H or I subunits (CHLH or CHLI) in N. benthamiana. Leaves 3 and 4 from 17-d-old N. benthamiana plants were sprayed with siRNAs formulated with the purified CD-5K fraction 5. The final concentration of siRNAs in the formulations was 12 ng μL⁻¹. Representative plants for each treatment are shown at 4 d after application. The application sites, leaves 3 and 4, are indicated by white arrows. A, Leaves that were sprayed with formulations containing a nontargeting siRNA. Scale bar = 5 cm. B, Leaves sprayed with a 22-mer siRNA targeting CHLI. C, Leaves sprayed with a 22-mer siRNA targeting CHLH. D, Transcript analysis of CHLH. At 5 d after treatment, leaf 4 from four plants was sampled for RT-qPCR analysis. Error bars represent the mean ± se. A Student’s t test (two-tailed) was used to compare transcript levels. The reduction in CHLH transcript was statistically significant at P = 3.48E−06.
are too large to efficiently diffuse through the plant cell wall. Based on their small size, we hypothesized that carbon dots might readily pass through the cell wall and provide an effective method for the delivery of siRNA into plant cells. Like other types of cationic transfection agents, amine-functionalized carbon dots can bind to the negatively charged polyphosphate backbone of nucleic acids. This binding provides protection from nucleases, and the shielding of negative charges can significantly enhance cellular uptake of nucleic acids (Kozielski et al., 2013).

Utilizing a microwave synthesizer, carbon dots were produced directly from bPEIs in a solution of chloroform and methanol. To make the technology more accessible to other labs, an alternative method of producing carbon dots from Glc and bPEIs in an aqueous solution and at a lower temperature was also developed. Carbon dots were prepared from several bPEIs of different Mr to yield a distribution of particle sizes that associated well with the size of the bPEI precursor. Purification of these preparations was an important step in optimizing the formulations—any components of the preparation that can bind RNA but are less efficacious for delivery will likely reduce the silencing activity by sequestering the siRNA. By screening fractions from the different preparations for silencing activity, highly efficacious carbon dot formulations were identified.

The size of the carbon dots appears to be an important variable in determining their efficacy. The carbon dots produced from the 25-kD bPEI precursors, with an average hydrodynamic diameter of 8.7 nm, had little silencing activity; this could be due to the size-exclusion limit of the cell wall. Somewhat unexpectedly, the smallest carbon dots that were prepared from the lower-Mr bPEIs also displayed limited silencing activity. The highest level of silencing was achieved with intermediate-sized carbon dots produced from the 5- or 10-kD bPEI precursors, with a hydrodynamic diameter near 3.9 nm. A small size may be a prerequisite for efficient delivery through the plant cell wall, but it does not appear to be the only factor affecting delivery and silencing efficiency of formulations. Subsequent barriers to delivery, such as endocytosis through the plasma membrane, escape from the endomembrane system, and release of siRNA, will also impact the silencing efficiency. Carbon dot characteristics that are optimal for passage through the cell wall may be
suboptimal for these later barriers. Further characterization of the carbon dots and studies aimed at interrogating delivery through the different barriers could allow for further improvements in silencing efficiency.

With a low-pressure spray application, the optimized carbon dot formulations showed good efficacy in the delivery of siRNA to N. benthamiana and tomato leaves. The targeting of a GFP transgene resulted in an almost complete loss of green fluorescence in both species. This gene-specific silencing was validated by measuring a >80% reduction in GFP transcript and protein levels. The efficacy of the carbon dot formulations was also demonstrated by targeting the H and I subunits of magnesium chelatase, which resulted in a visible bleaching phenotype and a significant reduction in the target gene transcript levels.

There have been several recent reports describing the use of nanomaterials for plant delivery and the silencing of transgenes (Mitter et al., 2017; Zhang et al., 2019), but a visible whole-leaf phenotype has not yet been reported. Leaf infiltration of a 21-mer siRNA linked with DNA-nanostructures resulted in silencing of a GFP transgene in N. benthamiana at 2 d post infiltration, but GFP protein levels recovered by 7 d post infiltration (Zhang et al., 2019). This transient silencing may be due to the use of a 21-mer siRNA, which produces less secondary siRNA than 22-mer siRNA. Clay nanosheets may enhance the stability of dsRNA but did not enhance the silencing of a β-glucuronidase reporter gene in Arabidopsis (Arabidopsis thaliana) relative to the dsRNA alone (Mitter et al., 2017). The carbon dot-facilitated delivery method for 22-mer siRNAs that is described here may have some advantages in that a strong, persistent, and visible phenotype can be achieved with a low-pressure spray application. The silencing of genes encoding the CHLH and CHLI subunits is also the first report of the silencing of endogenous plant genes with a nanomaterial-based delivery method. The simple spray application method and the strong silencing phenotypes achieved could make the current method a useful tool for plant functional genomics. Carbon dot formulations have recently been used to study the relationship between transgene expression levels, the production of secondary siRNA, and the systemic spread of silencing (B. Hendrix, P. Hoffer, R.A. Sanders, S.H. Schwartz, W. Zheng, B. Eads, D. Taylor, J. Deikman, unpublished data). Further improvements of nanomaterial delivery methods, such as carbon dots, could also have practical agricultural applications.

MATERIALS AND METHODS

Plants and Growth Conditions

The plants used in this study included the 16C GFP expression line of Nicotiana benthamiana (Voinnet and Baulcombe, 1997) and a tomato (Solanum lycopersicum) line with constitutive expression of an enhanced GFP (eGFP; Seminis Vegetable). Constitutive expression of eGFP in tomato was accomplished with the TraiMaker technology developed by Mendel Biotechnology (Ratcliffe et al., 2014). The HP375 line of tomato was transformed with a construct that contained a 35S promoter driving the expression of a LEXA DNA binding domain fused to a GAL4 activation domain. Another transgenic line was generated in the same tomato background with the eGFP transgene and an upstream LexA operator (opLEXA) sequence. The two lines were crossed and a line that was homozygous for both insertions was selected in a subsequent generation. Transactivation of the opLEXA–eGFP by the LEXA/GAL4 fusion protein resulted in constitutive expression of the eGFP reporter gene.

Plants were grown in 2.5-inch pots that were irrigated by an ebb-and-flow system with Peters professional 20-20-20 fertilizer. Plants were kept in a growth chamber maintained at 25°C with a light intensity of 150 μmol m⁻² s⁻¹ and a day length of 16 h.

Preparation and Purification of Carbon Dots

bPEIs with an average Mₙ of 1,200 D, 1,800 D, and 10 kD were obtained from Polysciences. The 25-kD bPEI was obtained from Sigma-Aldrich. The 5-kD bPEI, Lupasol G100, was supplied by BASF as a 50% (w/w) solution—before carbon dot preparation, the water was removed by lyophilization. Carbon dots were produced by a solvothermal reaction of the bPEIs in a solution of chloroform and methanol (4:1). Using a microwave synthesizer (Monowave 30, Anton Paar), 375 mg of bPEI in 5 mL of the solvent was heated to 155°C with a ramp time of 5 min and then held at 155°C for an additional 7 min. The reaction products were dried under nitrogen and then resuspended in water. Any residual chloroform was separated from the aqueous solution of carbon dots by centrifugation at 7,500 g for 5 min. The pH of the carbon dot solution was adjusted to 8.0 with 4N HCl, and the final volume was adjusted to 5 mL with water. For visible and fluorescence spectra, an aliquot was diluted in water (1:16). The absorption spectra were measured with standard 1-cm cuvettes in the cuvette port of a SpectraMax M2 microplate reader (Molecular Devices). The fluorescence spectra from the crude preparations and the fractions were measured in 96-well plates.

An alternative method was developed to produce highly efficacious carbon dots from Glc and 5-kD bPEI in an aqueous solution and at lower temperatures. For the PEI-functionalized Glc carbon dots, a 5-mL solution containing 37.5 mg mL⁻¹ Glc and 75 mg mL⁻¹ 5-kD bPEI was adjusted to pH 8.0 with 4N HCl and degassed under vacuum. The solution was then heated in a microwave synthesizer to 100°C over 3 min and then held at 100°C for an additional 5 min. The
visible spectra, fluorescence spectra, and FPLC chromatogram of the Glc carbon dots were similar to those of carbon dots produced directly from the 5-kD PEl.

FPLC Fractionation of Carbon Dot Preparations
Carbon dot preparations were purified by size-exclusion chromatography. A 2.5 × 20 cm Econo-column (Bio-Rad) filled with Sephadex G-50 superfine (GE Healthcare) was run on a Bio-Rad BioLogic Duo Flow FPLC system equipped with a QuadTec detector. The column was eluted at 2 mL min⁻¹ with 50 mM NaCl. Elution of the carbon dots was monitored at 360 nm. Five-milliliter fractions were collected starting after 30 mL.

Formulation of Carbon Dots
The final concentration of siRNA in the formulations was 12 ng mL⁻¹ or less. At higher concentrations of siRNA, aggregation can occur and a decrease in the silencing efficacy of formulations was observed. A similar observation was made with carbon dot formulations used to deliver plasmid DNA to animal cells (Pieratt et al., 2015). Pieratt et al. (2015) also observed that the size of the nanocomplexes decreased when the ratio of carbon dots to plasmid was high. The optimal concentration of carbon dots for gene silencing was determined empirically. A carbon dot:siRNA mass ratio of 40:50 worked well. The siRNA and the carbon dots were added separately to two tubes containing 10 mL MES buffer pH 5.7 and 20 mL glycerol. The two tubes were then combined with vortex mixing. Prior to spray application, the formulations were left at room temperature for at least 1 h. Longer incubations at room temperature (up to 1 week) did not significantly decrease the efficacy of the formulations (Supplemental Fig. S8).

Spray Application
The spreading surfactant BREAK-THRU S279 was added to a final concentration of 0.4% (v/v) within 1 h of spraying. The spray application was made with an Iwata HP M1 airbrush at a 12 pounds per square inch and with the fluid adjustment knob set to 3.5. With a slight depression of the airbrush trigger, a very light coat of the formulation was applied to the adaxial side of N. benthamiana leaves. The vertical growth habit of tomato allowed spray applications to the abaxial side of leaves, which allowed for better flooding and consequently improved gene silencing. The efficient stomatal flooding of the formulations was apparent by a subtle change in the shade of green. With this light spray application, there was no apparent damage to the leaves.

GFP Imaging and Analysis
The leaves were photographed using a custom-built imaging station equipped with a Canon EOS 70D camera with an EFS 18-55 mm macro 0.25-m/1.6 lens equipped with a Canon EOS Utility 2 software with tethered image acquisition. For GFP imaging, 58-mm Tiffen Green No. 11 and Yellow No. 12 filters were used on the camera. GFP fluorescence spectra, and FPLC chromatogram of the Glc carbon dots were similar to those of carbon dots produced directly from the 5-kD PEl.

For RT-qPCR analysis, total RNA was extracted from phenotypic leaf tissue collected 5 d after treatment using Trizol reagent (Invitrogen) according to the manufacturer’s protocol. The RNA was dissolved in water, and the concentration was measured using the Quant-iT RNA assay kit (Invitrogen). The total RNA samples were diluted to 5 ng mL⁻¹, and 50 ng of total RNA was used to synthesize random-primed, first-strand complementary DNA using the High-Capacity Reverse Transcription kit (Applied Biosystems). The reverse transcription products were used as a template for qPCR. The qPCR reaction mixtures consisted of 2 μL complementary DNA, 3 μL of a primer-probe mix (0.5 μM of each primer and 0.25 μM probe final concentration), and 5 μL Taqman Universal PCR Master Mix (Applied Biosystems). The sequences for the primer-probe sets are provided in Supplemental Table S3. The reactions were performed using an Applied Biosystems 7900HT Fast Real-Time PCR System with 40 cycles of two-step cycling at 95°C for 15 s and 60°C for 60 s. Target gene expression was expressed relative to a reference gene, Protein Phosphatase 2α (ScWv.1trP16930; http://benthamgene.cquat.edu.au/). Expression values were calculated using the comparative cycle threshold (Ct) method: 2⁻ΔCt Target – Ct Reference. A Student’s t test (two-tailed) was used to compare the transcript levels between formulations containing the non-targeting siRNA and the targeting siRNA.

For immunoblot analysis of N. benthamiana or tomato, whole leaves were harvested at 5 d after treatment, frozen, and then ground to a fine powder. Approximately 200 mg of ground tissue was homogenized in 300 mL of ice-cold buffer containing 20 mM Tris·Cl (pH 8.0), 150 mM NaCl, 0.1% (v/v) Triton X-100, and a protease inhibitor cocktail. The insoluble debris was removed by centrifugation. The protein concentration was quantified by Bradford assays, and 10 μg of total protein for each sample was run on a 12.5% Criterion Tris-HCl protein gel (4350014, Bio-Rad). Following electrophoresis, the proteins were transferred onto a polyvinylidene difluoride membrane and then blocked overnight with 5% skim milk in TBS plus 0.1% (v/v) Tween 20 (TBST). For GFP detection, a horseradish peroxidase-conjugated antibody directed against the full-length GFP protein (sc-8334, Santa Cruz Biotechnology) was diluted 1:1,000 in TBST and 5% (v/v) skim milk and incubated with the blot for 1 h. The blot was then washed four times with TBST for 10 min. Pierce ECL plus western substrate (32132, Thermo Scientific Pierce Protein Biology) was used for chemiluminescence detection of the GFP protein. ImageJ software was used to quantify band intensity for each sample. A Student’s t test (two-tailed) was used to compare the GFP protein levels between formulations containing the non-targeting siRNA and the GFP-targeting siRNA.

Accession Numbers
Accession numbers for the genes tested can be found in the legend of Supplemental Table S1.

Supplemental Data
The following supplemental materials are available.

Supplemental Figure S1. Spectroscopic characterization of carbon dots produced from the 5-kD bPEI.

Supplemental Figure S2. Binding and nuclease protection of dsRNA in carbon dot formulations.

Supplemental Figure S3. N. benthamiana at the time of treatment, 17 d after sowing.

Supplemental Figure S4. Silencing assays with unmodified 5-kD bPEI.

Supplemental Figure S5. The efficacy of CD-5K fractions from size-exclusion chromatography.

Supplemental Figure S6. Systemic silencing of GFP is initiated in the 16C line with carbon dot formulations.

Supplemental Figure S7. Persistence of the bleaching phenotype caused by CHLH silencing in N. benthamiana.

Supplemental Figure S8. Colloidal stability and efficacy of a carbon dot formulation with the 22-mer siRNA targeting CHLH.

Supplemental Table S1. siRNA sequences used in this study.

Supplemental Table S2. Dynamic light scattering measurements of the fractionated carbon dots from the CD-5K preparation prior to formulation with siRNA.
Supplemental Table S3. Oligonucleotide and probe sequences for RT-qPCR analysis of transcript levels.

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