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Nanoparticle carriers enhance RNA stability and uptake efficiency and prolong the protection against *Rhizoctonia solani*

Yumeng Wang[1,2†], Qin Yan[1,2†], Chi Lan[1,2], Tao Tang[1,2], Kuaibing Wang[3], Jie Shen[4] and Dongdong Niu[1,2*]

Abstract

Spray-induced gene silencing (SIGS) can inhibit plant diseases by topical application of double- (dsRNA) or single-stranded (sRNA) RNA molecules onto plants to silence virulence-related pathogen genes. However, the on-field application of SIGS is limited by the instability of naked RNA and low RNA uptake by pathogens. Nanoparticles have been used as RNA carriers to enhance RNA silencing. Rice sheath blight caused by *Rhizoctonia solani* (*R. solani*) is one of the most devastating fungal diseases in rice (*Oryza sativa* L.). In this study, we aimed to explore the protective effects of nanoparticle-delivered dsRNA against rice sheath blight. The key pathogenic genes, *RsAGO1* and *RsAGO2*, of *R. solani* were screened as targets for dsRNA. Chitosan (CS), polyethyleneimine (PEI), protamine, carbon quantum dot (CQD), polyamidoamine (PAMAM), and chitosan/SPc complex (CSC) were selected as dsRNA carriers. All the evaluated nanoparticles could assemble with dsRNA to form nanoparticle-dsRNA complexes, and CQD and CSC showed improved dsRNA load capacity. Particularly, CSC could enhance the stability of dsRNA and cause a 7% reduction in fluorescence intensity after nuclease treatment. CSC and CS effectively enhanced the efficiency of dsRNA uptake by pathogens. Furthermore, CSC could reduce pathogen infection and prolong the protection time of dsRNA by up to 20 days. Overall, this study provides a novel and efficacious SIGS-based strategy for producing RNA-based fungicides.

Keywords *Rhizoctonia solani*, RNA interference, Nanoparticles, Spray-induced gene silencing

Background

The productivity of major crops has been reduced by 20–30% worldwide due to plant pathogens and pests, threatening global food security (Savary et al. 2019). Notably, the extensive use of fungicides, necessitated by the emergence of pathogen resistance, increases harm to humans and the environment (Ma and Michailides 2005). Therefore, novel and eco-friendly strategies are urgently required for crop protection (Niño-Sánchez et al. 2022). RNA interference (RNAi) is a regulatory mechanism that triggers target gene silencing in almost all eukaryotes (Zamore et al. 2000; Iwasaki et al. 2015). RNAi plays an essential role in the growth, development, and gene expression of pathogens and pests (Huang et al. 2019); therefore, it can be used to control pathogens and pests (Zhang et al. 2017). Some pathogens and

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pests were shown to take up dsRNA from the environment (Whangbo and Hunter 2008; Wang et al. 2016; Qiao et al. 2021), which was termed 'Environmental RNAi' (Whangbo and Hunter 2008; Cai et al. 2018). This discovery led to the development of a potential method for disease control called spray-induced gene silencing (SIGS), which involves directly spraying plants with pathogen-targeting dsRNA or sRNA. These RNAs entered pathogen cells and silenced target genes to inhibit disease occurrence through environmental RNAi (Wang and Jin 2017; Cai et al. 2019; Qiao et al. 2021). Therefore, RNAi-based non-transgenic delivery systems such as SIGS facilitate pest and pathogen control by silencing target genes (Cagliari et al. 2019). Compared to conventional agrochemicals, 'RNA fungicides' targeting specificity could be more accurate (Cagliari et al. 2019). The foliar spraying application of dsRNA is also promising for developing commercially viable RNAi-based agricultural products (Jain et al. 2022). However, RNA is easily degraded in field soils and sediment water (Dubelman et al. 2014), limiting the on-field application of SIGS technology.

Nanoparticles are novel materials with a size range of 1–100 nm (Shidore et al. 2021), which have been found to play an important role in the enhanced efficiency of nanocarrier pesticides (Yan et al. 2021). The topical application of exogenous nanoparticle-dsRNA complexes on plants can improve the absorption efficiency of dsRNA into pathogens (Jain et al. 2022), which will reduce costs and improve the application efficiency of fungicides. Nanoparticles can be divided into three broad categories according to their properties: organic nanoparticles, inorganic nanoparticles, and carbon-based nanoparticles (Jat et al. 2020), and include liposome complexes (Taning et al. 2016), layered double hydroxide (LDH) clay nanosheets (Mitter et al. 2017), and carbon nanotubes (Demirer et al. 2020), respectively. Lipid-based nanocarriers that can deliver therapeutic siRNA/dsRNA drugs to mammalian cells (Wang et al. 2013) are expected to be developed for RNA delivery into plant pathogens. In addition, nontoxic and degradable LDH clay nanosheets can release exogenous dsRNA into plants and prolong the protection window against the virus (Mitter et al. 2017). The dsRNA loaded on LDH accumulates in the plant cells and is not easily washed off from the plant surface (Mitter et al. 2017). Additionally, an siRNA delivery platform based on carbon nanotubes can protect siRNA from nucleases and result in high RNA silencing efficiency in plant cells (Demirer et al. 2020). These results suggest that nanoparticles can promote RNA delivery efficiency and enhance the stability and durability of RNA, which have potential applications in SIGS.

Rice sheath blight caused by *Rhizoctonia solani* is a severe disease worldwide and causes 10–30% yield loss annually, even rising to 50% in endemic years (Singh et al. 2019). Therefore, developing safe, novel, and efficient strategies for plant disease control is important. In this study, we aimed to explore whether nanoparticle-delivered dsRNA could protect rice against sheath blight. We identified *RsAGO1* and *RsAGO2* as two crucial genes in *R. solani* that target key components of the RNAi pathway. To present a commercially viable and practical choice, we investigated six different nanoparticle-dsRNA complexes for their ability to protect the dsRNA from nucleases and facilitate penetration of the dsRNA into *R. solani*.

Results

SIGS using *RsAGO1/2*-dsRNA conferred resistance to rice sheath blight

Argonaute (AGO) proteins have been verified to directly guide sRNA to induce gene-silencing mechanisms, such as the degradation of target mRNA and heterochromatinization, in fungi (Siomi et al. 2011; Nguyen et al. 2018). Therefore, the abnormal expression of AGO proteins can disrupt sRNA-induced pathogenic mechanisms (Wu et al. 2020). In this study, *RsAGO1* and *RsAGO2* were screened as important RNAi target genes to form effective nanoparticle-dsRNA complexes against *R. solani*. The conserved domains of AGO proteins were highly homologous in the different anastomosis groups of *R. solani* (Fig. 1a). *RsAGO1* (ELU41296.1) and *RsAGO2* (ELU37743.1), encoding AGO-like enzymes in the *R. solani* AG-1 IA strain, were selected as candidate target genes. Next, we determined two segments from the non-conserved regions of *RsAGO1* and *RsAGO2*, containing 324 and 112 bp, respectively. Using reverse transcription-quantitative PCR (RT-qPCR), we found that relative transcript levels of *RsAGO1* and *RsAGO2* were significantly upregulated at 24 h after inoculating the stem of 4-week-old Nipponbare (NPB) seedlings with *R. solani* (Fig. 1b), indicating that these genes may play an important role in rice sheath blight and therefore, may be effective RNAi targets for the disease control.

To evaluate whether the downregulation of *RsAGO1/2* genes could effectively control *R. solani* infection, we synthesized an integrated *RsAGO1/2*-dsRNA fragment in vitro and analyzed it on a 1.5% agarose gel (Fig. 2a). Next, the *RsAGO1/2*-dsRNA was topically applied to detached rice leaves for disease assay. Results showed that *RsAGO1/2*-dsRNA treatments effectively reduced the lesion area of rice leaves compared with control treatments (Fig. 2b). Furthermore, we observed a significant reduction in the transcript levels of *RsAGO1/2* genes in *R. solani* at 24 and 48 h post-application of the dsRNA (Fig. 2c). Overall, these findings demonstrated that

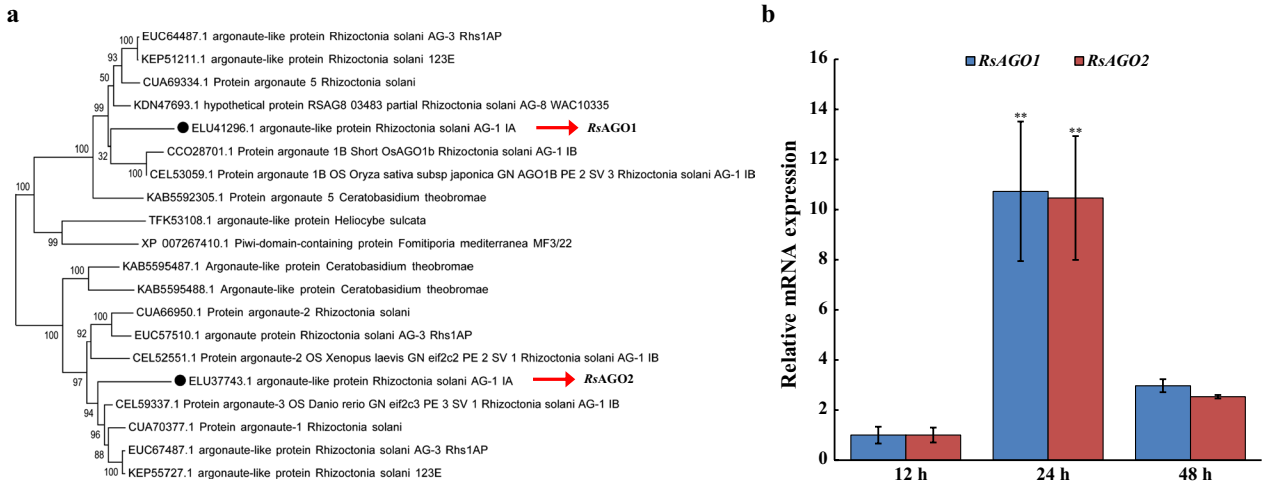

Fig. 1 Selection of target genes of *Rhizoctonia solani*. **a** The phylogenetic tree of the AGO gene family was performed using MEGA10.1.7 by the neighbor-joining method. **b** The stem of 4-week-old Nipponbare (NPB) seedlings was infected with *R. solani* mycelium plug (1 cm diameter). The transcript levels of candidate genes (*RsAGO1* and *RsAGO2*) were measured at 12, 24, and 48 h using RT-qPCR. Data are means ± SD of three replicates. **Indicates significant difference at $P < 0.01$ (Student's *t*-test)

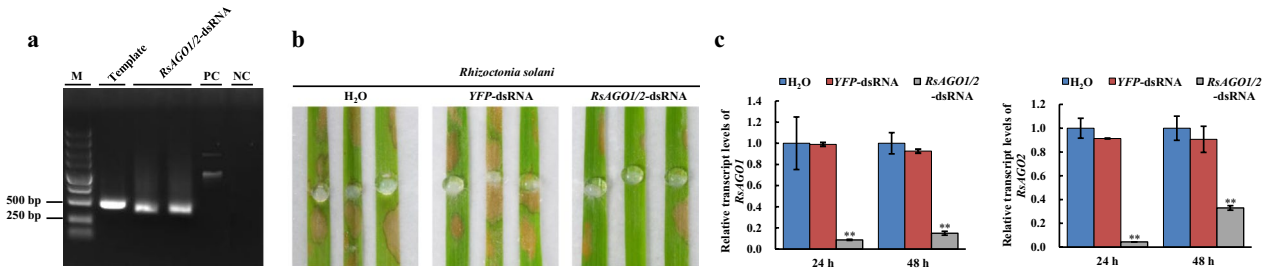

Fig. 2 Topical application of gene-targeting dsRNA synthesized in vitro inhibited the virulence of *Rhizoctonia solani*. **a** Synthesis of *RsAGO1/2*-dsRNA in vitro. Lane M: DL5000 DNA Marker; Lane Template: the DNA template of *RsAGO1/2*-dsRNA; Lane PC: replacing *RsAGO1/2* into control template in kit; Lane NC: system without T7 RNA Polymerase. **b** Rice leaves were inoculated with mycelium plugs of *R. solani* (5 mm diameter) and treated with *RsAGO1/2*-dsRNA on the same site (dsRNA: 50 ng/μL). Water and *YFP*-dsRNA were used as controls. **c** The impact of *RsAGO1/2*-dsRNA on the expression of *RsAGO1* and *RsAGO2* genes. Relative transcript levels of *RsAGO1* and *RsAGO2* were determined using RT-qPCR. Data are means ± SD from three replicates. Data are means ± SD of three replicates. **Indicates significant difference at $P < 0.01$ (Student's *t*-test)

externally applied *RsAGO1/2*-dsRNA protected plants from *R. solani* infection by silencing target genes.

Double-stranded RNA loading into nanoparticles to form spray complexes

To evaluate the capacity of the six selected nanoparticles to load dsRNA, we mixed *RsAGO1/2*-dsRNA with nanoparticles at various ratios, and the resulting nanoparticle-dsRNA complexes were incubated under different conditions. These complexes were analyzed via 1.5% agarose gel, and the dsRNA was loaded into nanoparticles completely when no dsRNA was migrating from the well in electrophoresis.

We found that *RsAGO1/2*-dsRNA was completely loaded into chitosan/SPc complex (CSC), chitosan (CS), carbon quantum dot (CQD), polyethyleneimine (PEI), polyamidoamine (PAMAM), and protamine at mass ratios of 1:5, 1:2, 1:60, 1:3, 1:2, and 1:2, respectively (Fig. 3). Collectively, the results suggested that naked dsRNA could be complexed with nanoparticles at varying mass ratios; CQD and CSC showed a better capacity to assemble with dsRNA.

Stability of dsRNA loaded into nanoparticles

The stability of dsRNA loaded into nanoparticles was evaluated using fluorescently labeled *YFP*-dsRNA. The protective effects of nanoparticles on dsRNA were assessed by measuring the changes in fluorescence intensity of the nanoparticle-dsRNA mixtures before and after the treatment with MNase (Micrococcal nuclease) (Fig. 4). We found that MNase reduced the fluorescence levels of *YFP*-dsRNA by 80%, whereas it reduced those of the dsRNA complexes

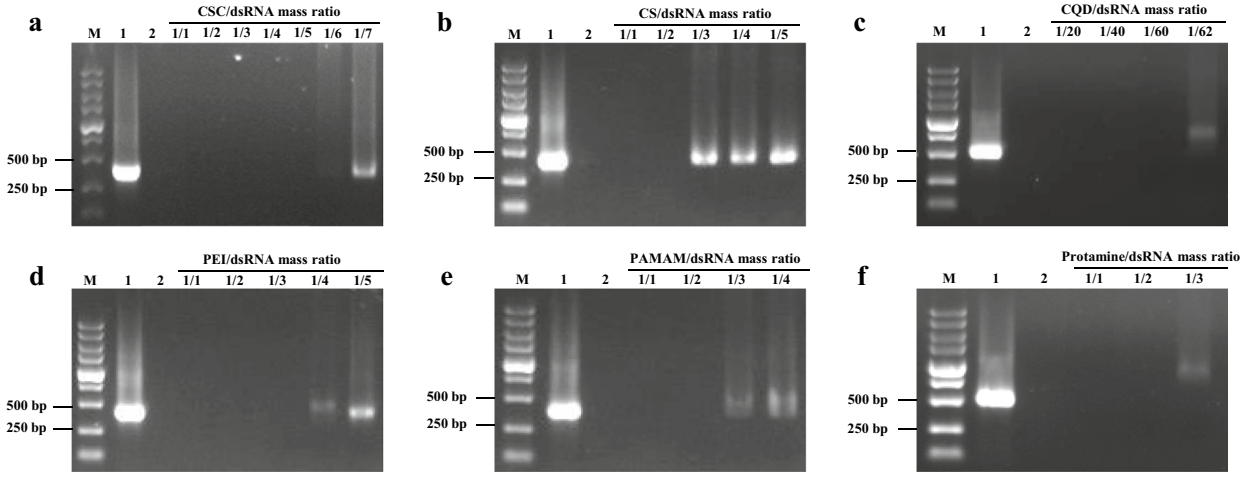

Fig. 3 The optimal mass ratios assay of nanoparticle-dsRNA complexes. **a**–**f** Gel electrophoresis assay of dsRNA retardation by nanoparticle-dsRNA complexes at varying mass ratios. Lane M: DL5000 DNA Marker; Lane 1: dsRNA only; Lane 2: nanoparticles only

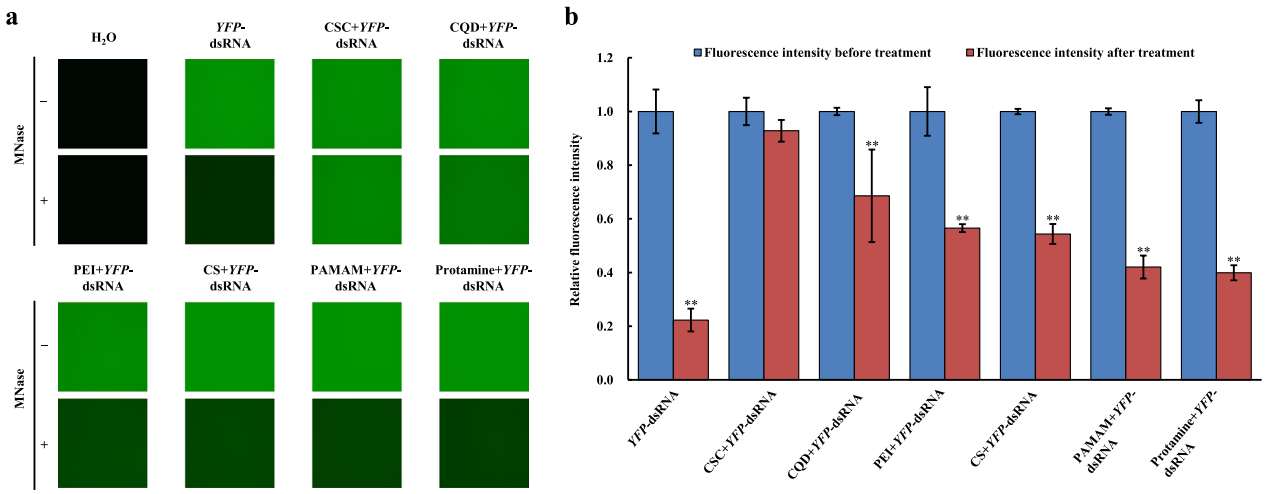

Fig. 4 Enhanced stability of dsRNA loading into nanoparticles. **a** Fluorescence signals of 5 μL reaction solutions (dsRNA: 50 ng/μL) and controls (water and *YFP*-dsRNA) before and after treatment with MNase for 30 min were analyzed using Zeiss inverted fluorescence microscope. Images of samples before and after treatment with MNase were taken, respectively. **b** Fluorescence intensities of nanoparticle-dsRNA complexes before and after treatment with MNase were assayed using ImageJ software. Data are means ± SD of three replicates. **Indicates significant difference at $P < 0.01$ (Student's *t*-test)

formed with CQD, PEI, CS, PAMAM, and protamine by 31, 43, 46, 58, and 60%, respectively (Fig. 4); notably, we observed no significant reduction in the fluorescence intensity of CSC-dsRNA complex after MNase treatments (Fig. 4). These results indicated that nanoparticles protected dsRNA from degradation, and CSC showed the best protection ability among those evaluated.

Enhanced uptake efficiency of nanoparticle-dsRNA complexes into pathogens

To explore whether nanoparticles could enhance the uptake of dsRNA into pathogens, we synthesized fluorescein-labeled *YFP*-dsRNA in vitro and co-incubated the complexes of labeled dsRNA-nanoparticles with *R. solani* on Potato Dextrose Agar (PDA) medium. The fluorescence signals in *R. solani* mycelia were measured using a Zeiss inverted fluorescence microscope. As shown in Fig. 5a, the pathogen could take up a low level of labeled dsRNA, and the hyphae's fluorescence is weak in the absence of nanoparticles. However, *R. solani* treated with the CSC-dsRNA and CS-dsRNA complexes showed highly increased fluorescence compared with the *YFP*-dsRNA control (Fig. 5a); the levels of relative fluorescence intensity were significantly

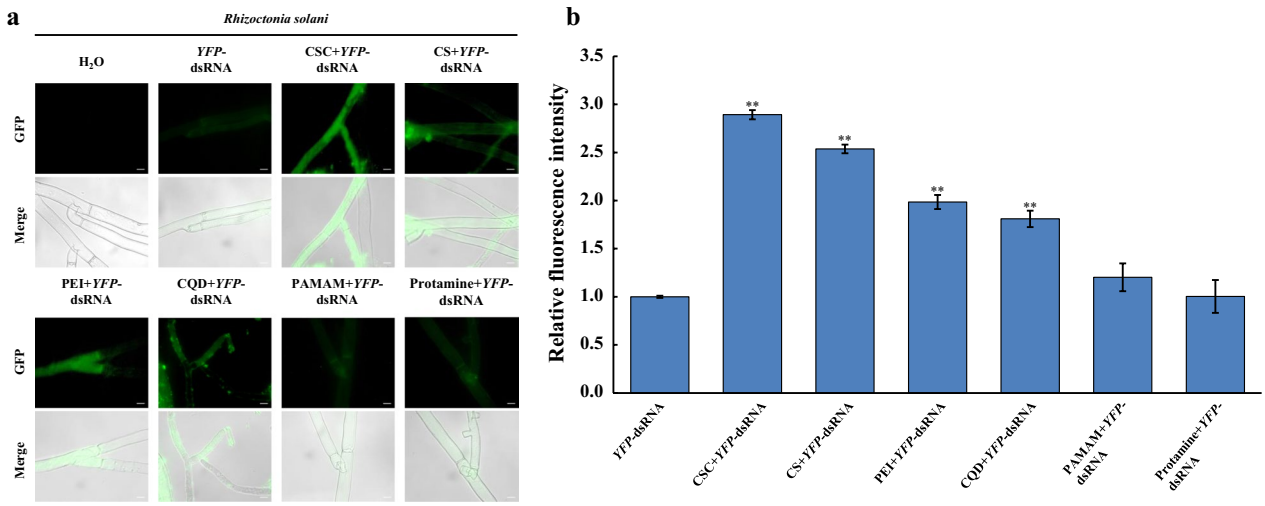

Fig. 5 Nanoparticle-dsRNA complexes improved the uptake of dsRNA by fungal cells. **a** *Rhizoctonia solani* mycelium plugs (5 mm diameter) and fluorescent *YFP*-dsRNA-nanoparticle complexes (dsRNA: 50 ng/μL) were co-incubated on PDA medium and the fluorescence signal was observed in the cytoplasm of *R. solani* cells. Samples were treated with MNase for 30 min before images were taken. Scale bars = 10 μm. **b** Relative fluorescence intensities were analyzed using ImageJ software. Data are means ± SD of three replicates. **Indicates significant difference at $P < 0.01$ (Student's *t*-test)

enhanced by over two-fold compared with *YFP*-dsRNA treatments (Fig. 5b). Furthermore, fluorescence signals were also obvious within *R. solani* mycelia treated with PEI-dsRNA and CQD-dsRNA, whereas mycelia treated with PAMAM-dsRNA and protamine-dsRNA had much lower fluorescence intensity (Fig. 5). These data demonstrated that nanoparticles assisted dsRNA uptake by pathogens, which substantially influenced the effectiveness of SIGS approaches.

Nanoparticles-*RsAGO1/2*-dsRNA complexes interfered with pathogen infection

To examine whether nanoparticles are more effective for SIGS strategies against *R. solani* in rice, we tested the ability of nanoparticle-*RsAGO1/2*-dsRNA complexes to prevent the disease. The detached rice leaves were inoculated with *R. solani* and simultaneously treated with the six nanoparticle-*RsAGO1/2*-dsRNA complexes at the inoculation sites. The results showed that various nanoparticle-*RsAGO1/2*-dsRNA complexes and *RsAGO1/2*-dsRNA resulted in more than a 53% reduction in lesion area compared to the control treatments with water, *YFP*-dsRNA, and nanoparticles (Fig. 6); strikingly, the CSC-*RsAGO1/2*-dsRNA complex was able to reduce the area of *R. solani* lesions by 92% in rice leaves (Fig. 6b). Overall, these results clearly demonstrated that externally applied nanoparticle-*RsAGO1/2*-dsRNA complexes significantly reduced the disease symptoms and protected rice plants from *R. solani* infection.

Nanoparticles prolonged the activity of dsRNA against *R. solani*

We examined whether CSC nanoparticles prolonged the protection window of *RsAGO1/2*-dsRNA against *R. solani* infection in rice plants. We inoculated detached leaves from rice plants with *R. solani*. Then we applied at inoculation sites with CSC nanoparticle-dsRNA complex or naked *RsAGO1/2*-dsRNA that had been incubated at 28°C for 0, 10, and 20 days. *YFP*-dsRNA, CSC, and water controls were set under the same conditions. Results showed that naked *RsAGO1/2*-dsRNA and CSC nanoparticle-dsRNA complexes of 0 days post treatment (dpt) inhibited the lesion development on rice leaves by 54% and 92% respectively, and those of 10 dpt reduced relative lesion area by 48% and 70%, respectively. Notably, the CSC-*RsAGO1/2*-dsRNA complex of 20 dpt reduced the relative lesion area by 54%, whereas the naked *RsAGO1/2*-dsRNA only resulted in a 32% reduction of lesion area (Fig. 7). Our data suggest that foliar application of the CSC-dsRNA complex may provide longer protection than naked dsRNA, which could improve the effectiveness of SIGS-based dsRNA in field applications.

Discussion

For effective RNAi against plant pathogens, essential genes are ideal lethal targets for pathogen inhibition and can be selected for SIGS. AGO proteins, the core component of RNA-induced silencing complexes (RISCs) (Faehnle and Joshua-Tor 2007), exist in almost all eukaryotic organisms and are highly conserved

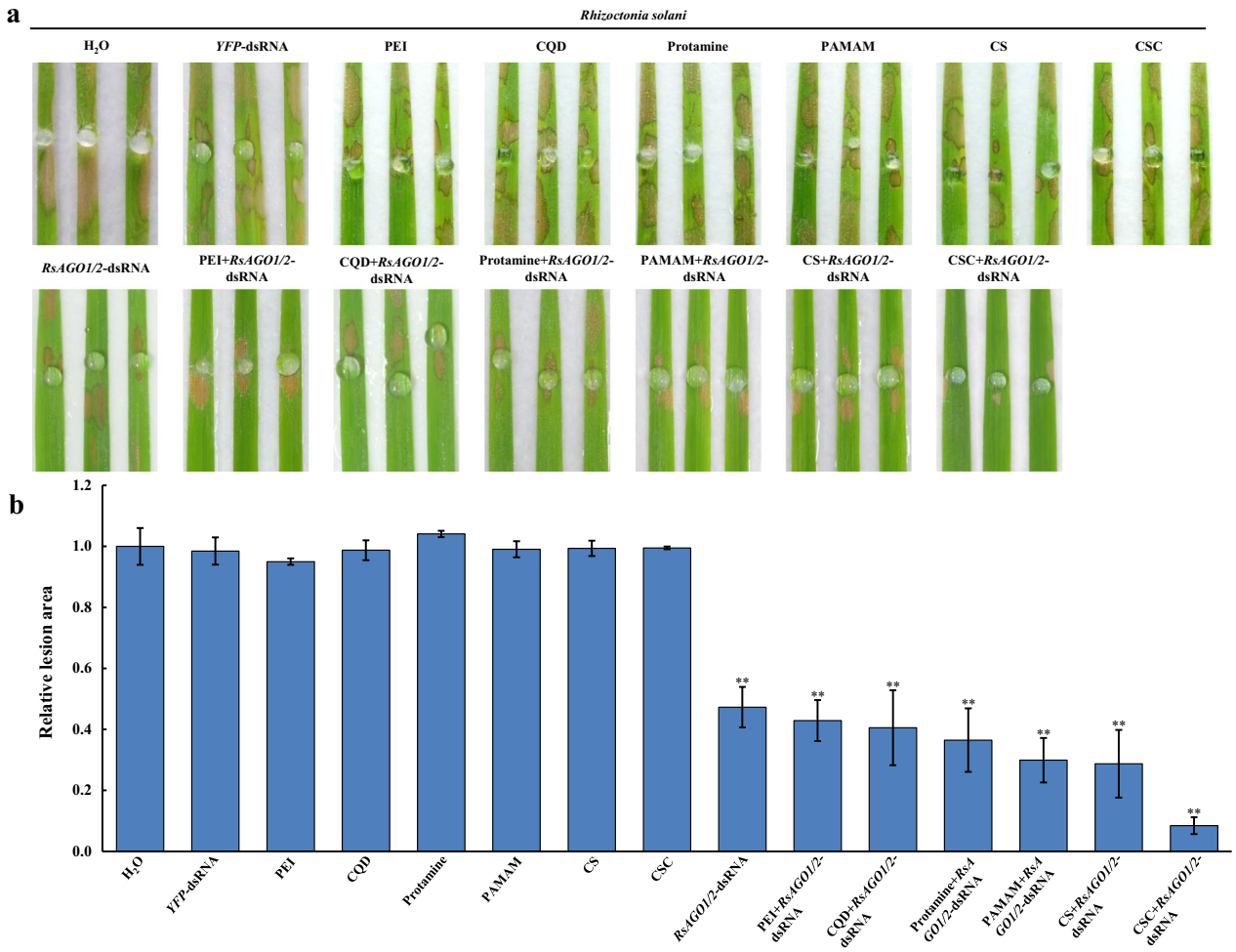

Fig. 6 Nanoparticle-dsRNA complexes improved the protection of rice plants against *Rhizoctonia solani*. **a** Detached rice leaves were inoculated by fresh *R. solani* mycelium plugs (5 mm diameter) and simultaneously treated with 20 μL of diverse nanoparticle-dsRNA complexes at the inoculation site for 48 h. The final concentration of dsRNA used in each treatment was 50 ng/μL. **b** Relative lesion area was measured and calculated using ImageJ software. The average value of the lesion area in water treatment is set to 1.0. Data are means ± SD of three replicates. **Indicates significant difference at $P < 0.01$ (Student's *t*-test)

(Stagsted et al. 2017). These complexes are involved in small RNA biogenesis and can regulate target gene expression in organisms (Wu et al. 2020). The AGO proteins play an important role in post-transcriptional gene silencing through cross-kingdom RNAi in pathogens (Wang et al. 2016; Cai et al. 2019), which can combine with small RNAs sent from host plants to silence virulence-related genes in the target pathogens (Huang et al. 2019; He et al. 2021). In this study, we determined *RsAGO1* and *RsAGO2*, which encode AGO-like proteins in *R. solani*, as candidate target genes. Considering the off-target effects, we selected and designed two gene segments from the non-conserved regions of *RsAGO1* and *RsAGO2* to minimize the potential biosafety risk brought by SIGS. In addition, the enhanced stability of dsRNA is closely related to the success of a SIGS approach and is essential for sustained crop protection (Joga et al. 2016). The half-life of dsRNA was suggested to be shorter than that of conventional agrochemicals under field conditions (Kupferschmidt 2013), and the in-soil concentration of dsRNA was found to decrease over time (Albright III et al. 2016; Fischer et al. 2016). Nanoparticles can help weaken these shortcomings (Landry and Mitter 2019) by reducing exposed acting sites in dsRNA for degradation by MNase (Ma et al. 2022), improving the adhesion of dsRNA onto plants (Kolge et al. 2021), and resisting ultraviolet radiation (Yang et al. 2022). We have demonstrated that nanocarrier CSC could provide robust protection for dsRNA from degradation by nucleases

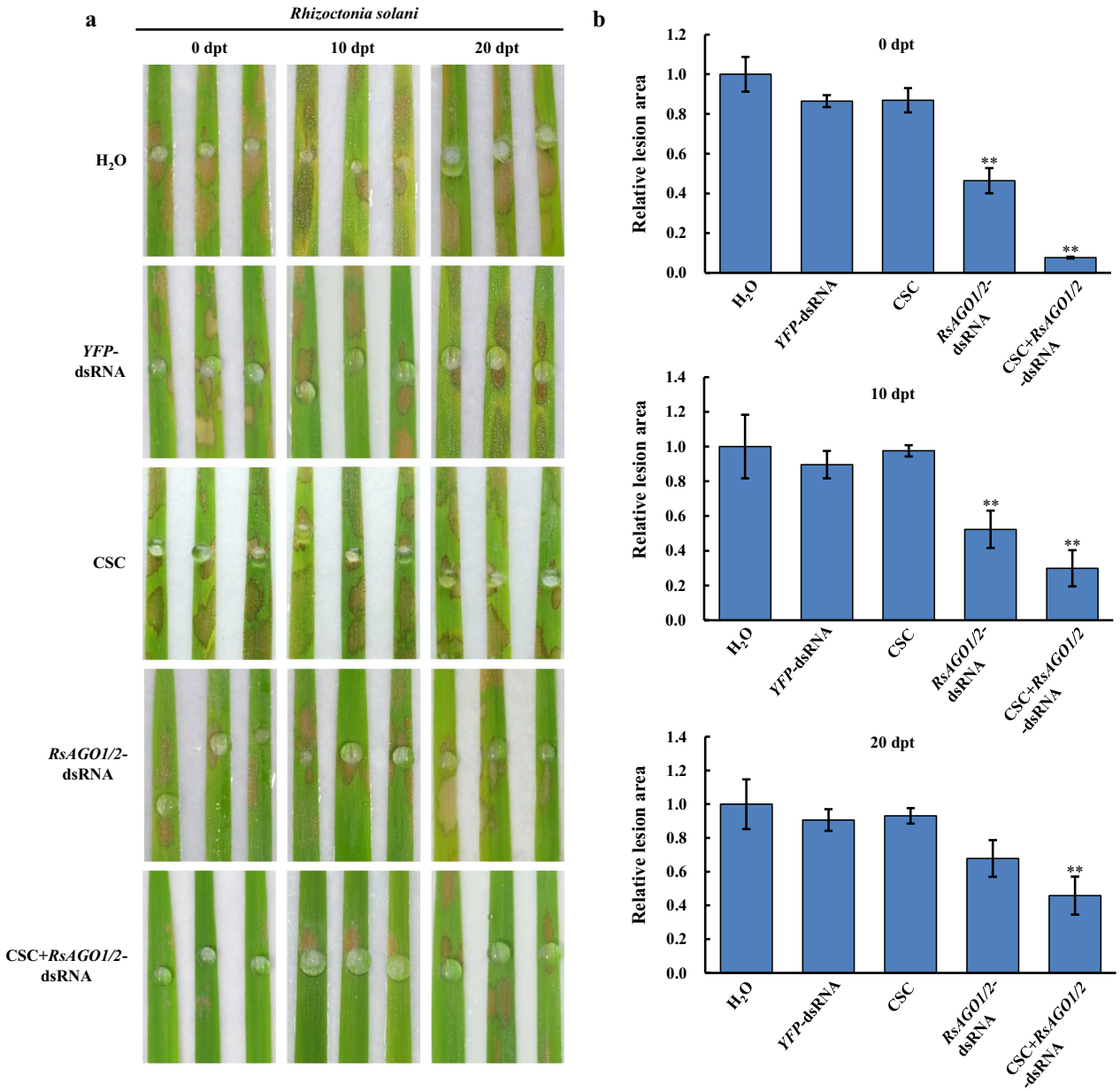

Fig. 7 CSC-dsRNA complex provides prolonged protection against *Rhizoctonia solani* in rice plants. **a** The leaves detached from rice plants were inoculated with actively growing *R. solani* mycelium plugs (5 mm diameter) and treated with naked *RsAGO1/2*-dsRNA or CSC nanoparticle-dsRNA complex (dsRNA: 50 ng/μL) that had been incubated at 28°C for 0, 10, and 20 days. **b** Relative lesion area was measured and calculated using ImageJ software. The average value of the lesion area in the water treatment is set to 1.0. Data are means ± SD of three replicates. **Indicates significant difference at $P < 0.01$ (Student's *t*-test)

(Fig. 4); thus, CSC can potentially be used to deliver dsRNA to control plant diseases.

A suitable delivery strategy is required for developing RNA pesticide products in agriculture. RNA delivery methods via foliar sprays, irrigation, nanocarriers, and trunk injection are feasible for pest control (Ghosh et al. 2017; Gogoi et al. 2017; Dalakouras et al. 2018; Yan et al. 2021), while successful cases of fungal infection control are limited. Our recent studies found that dsRNA uptake is efficient in numerous fungi, including *Botrytis cinerea*, *Sclerotinia sclerotiorum*, *Rhizoctonia solani*, *Aspergillus niger*, and *Verticillium dahlia* (Qiao et al. 2021). The use of nanoparticles as dsRNA carriers heightened the effectiveness of dsRNA uptake (Yong et al. 2021). In this study, we found that most of the dsRNA loaded into CSC and CS could enter *R. solani* mycelium, and the fluorescence

intensity increased by more than two times (Fig. 5). This observation indicates that nanoparticles improve the efficiency of dsRNA uptake by fungal cells.

Additionally, effective disease control and durability of exogenous dsRNA are important factors affecting the production cost in the market (Bocos-Asenjo et al. 2022). For dsRNA durability, the BioClay spray can extend the protection window for tobacco plants against viruses by up to 20 days compared to naked dsRNA (Mitter et al. 2017; Niño-Sánchez et al. 2022). In this study, CSC also prolonged the activity of dsRNA against *R. solani* for up to 20 days, with a 54% reduction in lesion area (Fig. 7). These results indicate that nanoparticle technology can assist in reducing disease symptoms and prolonging the window of protection against pathogen infection. Overall, our study showed that nanoparticles afforded enhanced RNA uptake efficiency and extended plant protection against fungal pathogen infection, which indicate that SIGS-based CSC-dsRNA delivery systems have the potential to be commercialized and provide feasible approaches for plant fungal disease management in the field.

Conclusions

We investigated the ability of six nanoparticle-dsRNA complexes to deliver dsRNA into fungal pathogens. Our findings demonstrate that topically applied dsRNA in nanoparticle-dsRNA complexes can trigger RNAi in *R. solani* and prolong RNAi-based protection against *R. solani*. The nanoparticle carriers tested in this study can enhance dsRNA stability and uptake efficiency and improve the effectiveness of the control of rice sheath blight disease. Our work indicates that nanoparticle-based delivery systems can mitigate the problems caused by RNA instability and low uptake and may thereby contribute to the translation of SIGS into disease control of field crops in the future.

Methods

Selection of target genes

To screen the target genes, a phylogenetic tree was constructed using MEGA10.1.7 software, and *RsAGO1* (ELU41296.1) and *RsAGO2* (ELU37743.1) in *R. solani* were selected as candidate target genes. The coding sequences of *RsAGO1* and *RsAGO2* in the *R. solani* AG-1 IA reference genome (Zheng et al. 2013) were accessed via the National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/), and the conserved regions of these two genes were obtained from NCBI (https://www.ncbi.nlm.nih.gov/Structure/cdd/docs/cdd_search.html). Next, we determined two segments from the non-conserved regions of *RsAGO1* and *RsAGO2*. We subsequently examined whether homologous genes were present in the host plants of *R. solani* to prevent the synthesized dsRNA from silencing homologous genes in the host plants.

In vitro synthesis of dsRNA and fluorescein-labeled dsRNA

To generate *RsAGO1/2*-dsRNA, the selected segments of *RsAGO1* and *RsAGO2* were PCR amplified with the primers listed in Additional file 1: Table S1. The *RsAGO1/2* fragment was generated by integrating two segments of *RsAGO1* (324 bp) and *RsAGO2* (112 bp) via overlap PCR and cloned into pMD19-T vector by TA cloning. The resulting construct was transformed into *Escherichia coli* (*E. coli*) DH-5α, and the clone with the correct sequence was used for subsequent RNAi experiments. The in vitro Transcription T7 Kit (TaKaRa, Beijing, China) was used to synthesize *RsAGO1/2*-dsRNA by in vitro transcription from both 5' and 3' ends of the *RsAGO1/2* construct (Additional file 1: Table S2) in a 20 μL reaction. The *RsAGO1/2*-dsRNA was purified to be used for subsequent RNAi experiments. Fluorescein-labeled dsRNA was synthesized using a fluorescein RNA Labeling Mix Kit (Sigma-Aldrich, St. Louis, MO, USA), and the fluorescent dsRNA was used directly.

Plant growth conditions and pathogen infection

NPB plants were cultivated in a growth room at 28°C and 70% relative humidity with 14-h light (200 μE m^{-2} s^{-1}) /10-h dark cycles.

The fungus *R. solani* was grown at 28°C for 48 h on PDA medium. To evaluate transcript levels of candidate genes (*RsAGO1* and *RsAGO2*) after *R. solani* inoculation, a mycelium plug (1 cm diameter) of actively growing *R. solani* was placed onto the stem of 4-week-old rice seedlings after spraying the infection site with ddH_2O to maintain humidity. The inoculated rice plants were placed in an infection box in a greenhouse at 28°C and 80% relative humidity and were first cultivated in the dark for 12 h, followed by 12-h light /12-h dark cycles. The samples were collected at 12, 24, and 48 h post inoculation.

The SIGS-based rice plants protection assay was performed using detached leaves. Third leaves were detached from 4-week-old rice seedlings and inoculated with mycelium plugs (5 mm diameter) of actively growing *R. solani*, and 20 μL of synthesized dsRNA (50 ng/μL) was applied onto the inoculation site. Inoculated rice leaves were incubated at 28°C and 80% relative humidity in the dark for 12 h, followed by 12-h light/12-h dark cycles.

Preparation of nanoparticle-dsRNA complexes

The CS-dsRNA complex was prepared as previously described (Zhang et al. 2010). The CS (Sigma-Aldrich,

St. Louis, MO, USA) was dissolved in the buffer (0.1 M HOAc, pH 4.5) to obtain a 0.1 g/100 mL CS working solution. The CS-dsRNA complex was assembled by mixing dsRNA with varying amounts of CS (CS:dsRNA from 1:1 to 1:5 (w/w)). The mixture was incubated in a water bath at 55°C for 1 min and then vortexed for 30 s via a high-speed vortex. The complete loading of dsRNA was estimated via 1.5% agarose gel electrophoresis when no dsRNA was observed migrating into the gel.

The PEI-dsRNA complex was prepared as described (Feng et al. 2017). The PEI (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in the buffer (0.1 M HOAc, pH 4.5) to make a 0.1 g/100 mL PEI working solution. The PEI-dsRNA complex was assembled by mixing dsRNA with varying amounts of PEI (PEI:dsRNA from 1:1 to 1:5 (w/w)). The mixture was vortexed for 1 min via a high-speed vortex and incubated at 25°C for 5 min. The optimal ratio between the mass of nanoparticles and dsRNA was assessed as described above.

The protamine-dsRNA complex was prepared as described (Rengaswamy et al. 2016). Protamine (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in 20 mmol/L HEPES buffer (pH 7.4) to make a 0.03 g/100 mL protamine solution. The protamine-dsRNA complex was assembled by mixing dsRNA with varying amounts of protamine (protamine:dsRNA from 1:1 to 1:3 (w/w)). The mixture was vortexed for 5 min via a high-speed vortex and incubated at room temperature for 30 min. The optimal ratio between the mass of nanoparticles and dsRNA was assessed as described above.

The PAMAM-dsRNA complex was prepared as described (Lu et al. 2019). The PAMAM (Weihai CY Dendrimer Technology Co., Ltd.) was dissolved in DEPC·H_2O to make a 0.1 g/100 mL PAMAM solution. The PAMAM-dsRNA complex was assembled by mixing dsRNA with varying amounts of PAMAM (PAMAM:dsRNA from 1:1 to 1:4 (w/w)). The mixture was vortexed for 1 min via a high-speed vortex and incubated at room temperature for 5 min. The optimal mass ratio test was performed as described.

The CQD-dsRNA complex was prepared as described (Das et al. 2015). The CQD was fabricated with the assistance of the microwave method. Subsequently, 9 mL polyethylene glycol (PEG-200, Sigma-Aldrich) and 100 mg polyethyleneimine (PEI) were added to 3 mL DEPC·H_2O and 2 mL DEPC·H_2O, respectively, and the mixtures were microwaved at 800 W for 3 min. The dsRNA was assembled with varying amounts of CQD (CQD:dsRNA from 1:20 to 1:62 (w/w)), and the mixture was vortexed for 1 min via a high-speed vortex and incubated at 4°C overnight. The optimal mass ratio was assessed as described above.

The CSC-dsRNA complex was prepared as described (Wang et al. 2021). CSC (≥ 90% degree of deacetylation) was dissolved in a 1% HOAc solution to obtain a 0.2% CS solution, and SPc was dissolved in ddH_2O to obtain a 0.2% SPc solution. The 0.2% CS solution was mixed with the same volume of 0.2% SPc solution and incubated at room temperature for 15 min to prepare a 0.1% CSC solution (China Agricultural University). The CS particles could combine with SPc in the solution to form CSC complex. To prepare the CSC-dsRNA complex, the dsRNA was assembled with varying amounts of CSC (CSC:dsRNA from 1:1 to 1:7 (w/w)) and mixed by vortexing for 1 min via a high-speed vortex and incubated at room temperature for 5 min. The optimal mass ratio test was performed as previously described.

Stability assay of nanoparticles-dsRNA complexes

To assess the stability of dsRNA in nanoparticle-dsRNA complexes, fluorescein-labeled *YFP*-dsRNA was synthesized and used to prepare nanoparticle-dsRNA complexes (dsRNA: 50 ng/μL). Each resultant sample (5 μL) was mixed with MNase (Thermo Fisher Scientific, Waltham, MA, USA) and incubated for 30 min at room temperature. An inverted fluorescence microscope was used to observe the fluorescence of dsRNA in nanoparticle-dsRNA complexes before and after treatment with MNase. To investigate the stability of dsRNA, dsRNA was assembled with nanoparticles at the optimal mass ratio, and the changes in the complex's fluorescence before and after treatment with MNase were analyzed using ImageJ software.

Assay of dsRNA uptake by *R. solani*

A fluorescein-labeled *YFP*-dsRNA and nanoparticle mixture (*YFP*-dsRNA: 50 ng/μL) was prepared at the optimal mass ratio. Twenty microliters of the solution of each nanoparticle-*YFP*-dsRNA complex were added to *R. solani* mycelium plugs (5 mm diameter) and placed on a microscope slide containing 4 mL PDA medium. The mixture was incubated in the dark at 28°C for 12 h, and then MNase was used to treat the mycelium at 37°C for 30 min to degrade the unabsorbed *YFP*-dsRNA. The fluorescence in *R. solani* mycelia was observed using an inverted fluorescence microscope. The relative fluorescence intensities of *R. solani* mycelia were analyzed using ImageJ software.

Total RNA extraction and RT-qPCR

The samples of stem or detached rice leaves inoculated with *R. solani* were collected and frozen at −80°C. Total RNA was extracted using the TRIzol extraction method and treated with DNase I (Thermo Fisher Scientific, Waltham, MA, USA) to remove genomic DNA. The

cDNA was synthesized using the Superscript™ III First-Strand Synthesis System (Thermo Fisher Scientific, Waltham, MA, USA), and RT-qPCR was performed using the 2 × AceQ® qPCR SYBR® Green Master Mix (Vazyme, Nanjing, China). The reaction conditions for RT-qPCR were as follows: 95°C for 5 min, 40 cycles at 95°C for 10 s and 60°C for 34 s, followed by 95°C for 15 s, 60°C for 60 s and 95°C for 15 s for PCR melt curve. For the RT-qPCR of rice, 18S rRNA was used as a reference gene. The primers used in this study are listed in Additional file 1: Table S1.

Abbreviations

AGO	Argonaute
CQD	Carbon quantum dot
CS	Chitosan
CSC	Chitosan/SPc complex
dpt	Days post treatment
dsRNA	Double-stranded RNA
E. coli	*Escherichia coli*
HEPES	2-[4-(2-Hydroxyethyl)piperazin-1-yl]ethanesulfonic acid
LDH	Layered double hydroxide
miRNA	MicroRNA
MNase	Micrococcal nuclease
NPB	Nipponbare
PAMAM	Polyamidoamine
PBS	Phosphate buffered saline
PEG	Polyethylene glycol
PEI	Polyethyleneimine
RNAi	RNA interference
R. solani	*Rhizoctonia solani*
RT-qPCR	Reverse transcription-quantitative PCR
SIGS	Spray-induced gene silencing
siRNA	Small interfering RNA
SPc	Star polycation
sRNA	Small RNA

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s42483-023-00157-1.

Additional file 1: Table S1. PCR primers used in this study. **Table S2.** The sequences of dsRNAs in this study.

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Authors' contributions
DN conceived the project. YW, QY, and CL performed the experiments. YW, QY, TT, KW, JS, and DN wrote the manuscript. All authors read and approved the final manuscript.

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Declarations

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Not applicable.

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The authors declare that they have no competing interests.

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