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Targeted gene suppression through double-stranded RNA application using easy-to-use methods in *Arabidopsis thaliana*

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Abstract

RNA interference (RNAi) is an RNA-dependent gene silencing process that is regulated by the interaction between the RNA-induced silencing complex (RISC) and double-stranded RNA (dsRNA). Exogenous dsRNAs are imported directly into the cytoplasm, where they are cleaved by Dicer into short dsRNA fragments of 20–25 base pairs. These short dsRNA fragments, called small interfering RNAs (siRNAs) have sequence-specific interaction with target genes. The guide strand, onto which siRNAs are incorporated in the RISC interacts with the target mRNA sequence, thereby inducing cleavage and degradation of target messenger RNAs (mRNAs) by ribonucleases. Recent studies have shown that plant dsRNA treatments can induce RNAi. However, the dsRNA application methods and delivery systems involved have not been well examined. In this study, dsRNA was introduced to *Arabidopsis thaliana* by two methods: dipping and spray. We synthesized two dsRNAs designed to target mRNAs encoding enhanced green fluorescent protein (*EGFP*). After applying dsRNAs that target *EGFP*, we found an obvious reduction in GFP expression. This was determined using fluorescence microscopy and quantitative reverse transcription PCR to assess the mRNA levels of the auxin-sensitive reporter *DR5-EGFP Arabidopsis thaliana*. Our data revealed that applying target gene-specific exogenous dsRNAs can induce suppression of target genes of interest whether the dipping or spray method is used. This study therefore provides a foundation for understanding how to apply and deliver dsRNAs in plants.

Keywords: *Arabidopsis thaliana*, Double-stranded RNA, Exogenous RNA, RNA interference

Introduction

RNA interference (RNAi), a process that based on small interfering RNA (siRNA) has been employed to regulate plant growth, plant stress tolerance and other plant processes by inhibiting the expression of particular genes [1]. In plants, double-stranded RNA (dsRNA) can induce RNAi; this is a biological gene silencing process capable of using sequence-specific gene targeting to inhibit gene translation or transcription [1]. The dsRNAs are cleaved by a ribonuclease called Dicer-like enzyme (DCL) [2] to produce small non-coding RNAs called siRNAs. Each

siRNA is unwound into two single-stranded RNAs (ssRNAs), known as the passenger strand and the guide strand. The passenger strand is degraded, while the guide strand associates with Argonaute proteins (AGOs) [3] to form the RNA-induced silencing complex (RISC) [3]. The RISC drives the silencing of target mRNAs via sequence-specific base pairing, ultimately resulting in mRNA degradation and translation repression [4, 5].

RNAi-based technologies have been applied in plant systems to control gene regulation, and to mitigate risk associated with exposure to viruses, viroids, fungi, insects, mites, and nematodes [6, 7]. Previous studies have reported that it is difficult to apply dsRNA or siRNA without accessory techniques (i.e., using a protein carrier, or a clay nanosheet and a high-pressure spray) [8–10]. Despite these complications, few studies have focused on

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the effects of various dsRNA sequences and their effective concentrations with respect to target gene suppression and the location of dsRNA delivery in plants.

Green fluorescent protein (GFP) is frequently used as a reporter of gene expression and transcriptional activity, both in single cells and in tissues. Because GFP expression can be monitored visually, it can be used to identify when proteins are made and where these proteins are located in cells or tissues [11, 12]. In addition, GFP expression is easily imaged, so it is easy to quantify the expression level of GFP-linked mRNAs. Therefore, we took advantage of *enhanced GFP (EGFP)*-expressed transgenic *Arabidopsis* to study the action of dsRNAs targeting *EGFP* mRNA delivery in plants.

In this study, we suppressed the gene expression of *EGFP* by direct dipping or by a spray protocol to examine the effectiveness of these dsRNA treatment methods. Two dsRNAs were synthesized to target *EGFP* mRNAs, which divided the mRNA into two pieces. In addition, we also varied the concentration of dsRNA during dsRNA application. Taken together, our results suggest that both the dsRNA sequence and its application concentration are important determinants of target gene suppression. We hope our results help to provide a foundation for RNAi-based plant technologies involving dsRNA.

Materials and methods

Plant materials and growth conditions

We used two *Arabidopsis* accessions for this study: wild-type Columbia-0 (Col-0) and an auxin-responsive fluorescent marker *DR5-EGFP* mutant. Both Col-0 and *DR5-EGFP* seeds were surface-sterilized with 30% (v/v) bleach for 10 min and then washed five times with sterile distilled water (dH₂O). Col-0 seeds were plated on plates containing autoclaved 0.215% Murashige and Skoog (MS) medium basal salts mixture (Duchefa, Netherlands), 0.5 g/L MES (Duchefa, Netherlands), 1% sucrose (Junsei, Japan), and 0.7% phyto agar (Duchefa, Netherlands) growth medium, pH 5.7. *DR5-EGFP* seeds were plated and selected on the above MS media that also contained 25 mg/L hygromycin (Duchefa, Netherlands). Seedlings were then grown at 23 °C with 16 h of light per day in a growth chamber.

Double-stranded RNA design and synthesis

The dsRNAs were designed to be approximately 500 base pair (bp) in length and to target the 5' and 3' regions of the *EGFP* coding sequence (Fig. 1a). Our dsRNA synthesis used DNA templates of designed dsRNA regions that included T7 promoter sequences (5'-TAATACGAC TCACATATAAGAGAG-3'). These DNA templates were synthesized by polymerase chain reaction (PCR) using

Phusion High-Fidelity DNA polymerase (Thermo Scientific, United States) used as per the manufacturer's protocol. PCR products were further developed to synthesize dsRNAs using the MEGAscript RNAi Kit (Invitrogen, United States) as per the manufacturer's protocol (Additional file 1: Fig. S1). The quality of the dsRNAs was determined using gel electrophoresis on a 1% agarose gel (Additional file 1: Fig. S2). The PCR primers we used are listed in Additional file 1: Table S3.

Dipping and spray methods of dsRNA application

We applied dsRNAs to 1-week-old *Arabidopsis* seedlings by dipping and by spray. The dipping method was performed as follows: 1-week-old *Arabidopsis* plants were dipped in a 24-well plate with sterilized water containing dsRNAs at different concentrations (i.e., 5, 10, 20, or 40 ng/μL). For the spray method, sterilized water containing dsRNAs (in concentrations of 5, 10, 20, or 40 ng/μL) was sprayed onto 1-week-old *Arabidopsis* using a sterilized sprayer. Next, whole *Arabidopsis* seedlings were placed in sterilized water containing dsRNAs for 1, 2, 4, and 6 days if using the floral dip method, or were placed in soil for 1, 2, 4, and 6 days after dsRNA treatment if the spray method was used. On each day post-treatment (dpt), the whole *Arabidopsis* plant was washed five times with sterilized water to remove dsRNAs from the surface of plants. Plants were then sampled. For the fluorescence expression experiment, after each dpt the *DR5-EGFP Arabidopsis* plant was dipped in sterilized water containing 1 μM 1-Naphthaleneacetic acid (NAA; Sigma-Aldrich, United States) for 24 h and was then washed five times with sterilized water before being sampled.

Fluorescence microscopy

For fluorescence microscopy, the roots of *Arabidopsis* plants were mounted in distilled water between a 48 × 60 and a 24 × 60 cover slip. Imaging was performed on a Universal Fluorescence Microscope (Carl Zeiss, Germany). EGFP emission was detected using a 520–525 nm band pass filter and a 100× lens. Image analysis was performed in ZEN version 2.3 (Carl Zeiss).

Total RNA extraction

For total RNA extraction, whole plants were sampled at each dpt. *Arabidopsis* samples were first homogenized in liquid nitrogen. Total RNA isolation was performed using TRIzol Reagent (Invitrogen) and extractions were treated with Recombinant DNase I (Takara, Japan) to eliminate single- and double-stranded DNA. All procedures were performed as per the manufacturer's instructions.

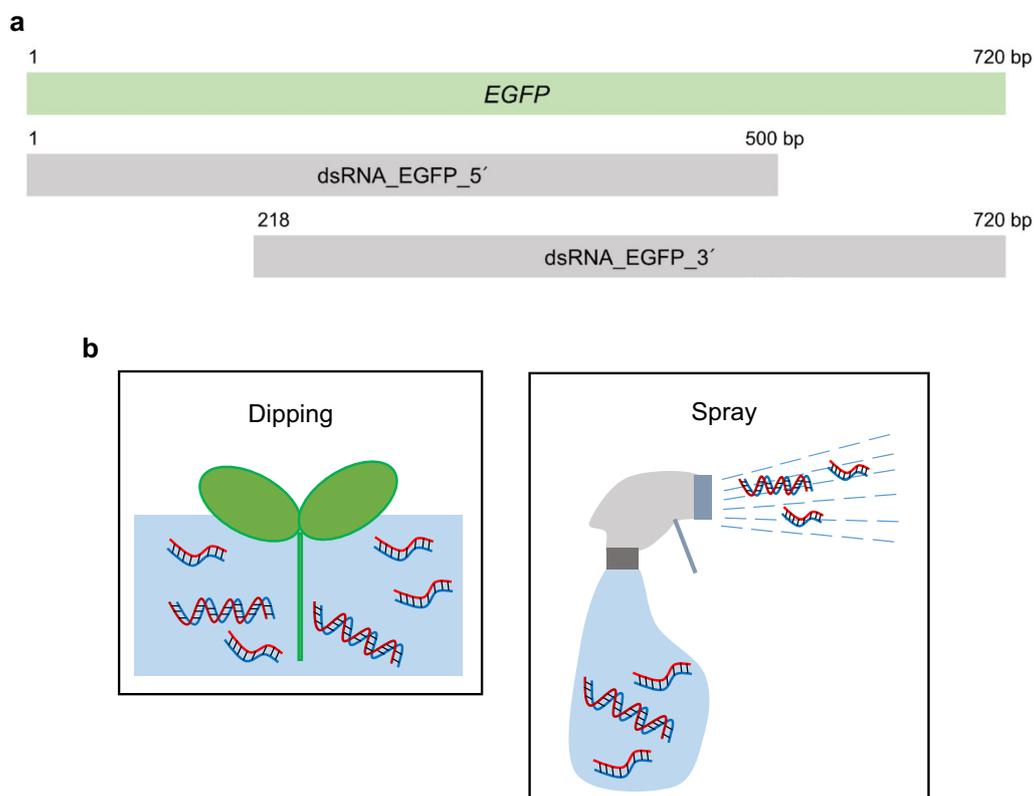


Fig. 1 Design of dsRNAs and application methods. **a** The dsRNA_EGFP_5' and dsRNA_EGFP_3' dsRNAs, both of which were approximately 500 bp in length, were designed to target the 5' and 3' regions of the *EGFP* mRNA, respectively. **b** Dipping and spray methods for the direct application of dsRNAs onto plants

Quantitative real-time PCR

Complementary DNA (cDNA) was synthesized from each RNA sample using SuperScript III Reverse Transcriptase (Invitrogen). Reverse transcription was performed using 2 μ g of total RNA and oligo (dT) primer (Thermo Scientific), as per the manufacturer's instructions. For determinations of the effective concentration of dsRNA, cDNA was synthesized using random hexamer primers (Thermo Scientific). Quantitative real-time PCR (qRT-PCR) was performed using Light Cycler 480 SYBR Green I Master Mix (Roche, United States) with SYBR Green detection and gene-specific primers. Ct values for genes were obtained using At3g18780 (*AtACT2*) as a control, and relative expression values were calculated using the $\Delta\Delta$ Ct method. All primer sequences used are listed in Additional file 1: Table S4.

Results

Design and synthesis of dsRNAs targeting *EGFP*

Our *EGFP* line was used to study whether dsRNAs that specifically target *EGFP* were properly delivered and

effective in *Arabidopsis*. When, in the presence of dsRNAs, the expression of *EGFP* is suppressed, it can be visually monitored by fluorescence measurements. Therefore, we designed two dsRNAs to determine which were most effective in suppressing the *EGFP* coding sequence (CDS). These two dsRNAs, both of which were approximately 500 bp, were designed to target the 5' and 3' regions of the *EGFP* CDS (hereafter referred to as dsRNA_EGFP_5' and dsRNA_EGFP_3', respectively) (Fig. 1a). We used NCBI BLAST to design dsRNA sequences that are present only in the *EGFP* CDS. In addition, we checked which off-target effects could be expected from the designed dsRNAs sequences using the dsCHECK tool [13]. The expected off-target effects of the designed dsRNAs are presented in Additional file 2: Table S1 and Additional file 3: Table S2. Next, we synthesized two kinds of dsRNAs to assess their effects on the target genes. The purity of our synthesized dsRNAs was confirmed using gel electrophoresis (Additional file 1: Figure S2).

Determination of the effective concentration of dsRNAs to apply into *Arabidopsis thaliana* using dipping and spray methods

We used two dsRNA application methods, dipping and spray methods, with effective concentration of dsRNAs without additional techniques to regulate the expression of *EGFP* (Fig. 1b). To determine the effective final concentration of dsRNAs for these applications, we diluted the synthesized dsRNA_EGFP_5' in water to concentrations of 5, 10, 20, or 40 ng/μL. We then added 2 mL of each diluted dsRNA_EGFP_5' to 1-week-old Col-0 seedlings using the dipping and spray methods (thereby resulting in a total 1, 2, 4, or 8 μg of dsRNA per plant; each sample contained ten plants). Since the *EGFP* coding sequence was not present in the Col-0 genome, the direct effect of dsRNA concentration could be analyzed using qRT-PCR products amplified with a dsRNA_EGFP_5' specific primer from 1, 2, 4, or 6 dpt plant samples. We found the

highest levels of dsRNA_EGFP_5' expression in 1 dpt plants treated with 20 ng/μL dsRNA_EGFP_5' by dipping and 2 dpt plants treated with 20 ng/μL dsRNA_EGFP_5' by spray. The 20 ng/μL concentration of dsRNA_EGFP_5' was effective in plants treated using both the dipping and spray methods. (Fig. 2a, b). These results showed that the concentration is more important than the total amount of dsRNA applied to plants. In addition, the delivery of exogenous dsRNA to plants using the dipping method is expected to be faster than delivery using the spray method. For this reason the 20 ng/μL dsRNA concentration was used for subsequent experiments.

Suppression of fluorescence and *EGFP* mRNA expression using *EGFP*-specific dsRNAs

One-week-old *DR5-EGFP* transgenic *Arabidopsis* plants were surface treated with exogenously synthesized

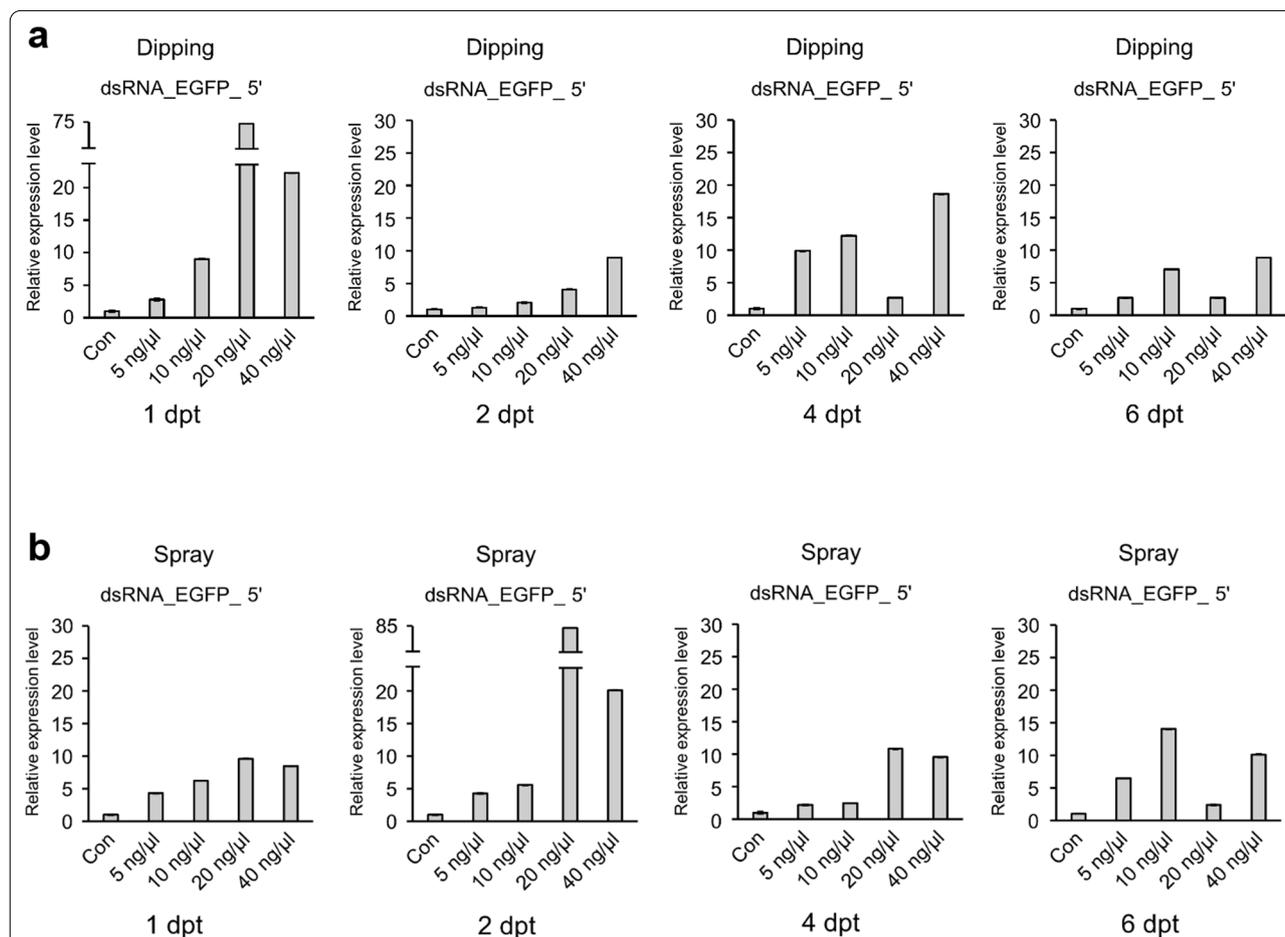


Fig. 2 Determination of effective dsRNA concentrations for dipping and spray methods. **a, b** Synthesized dsRNA_EGFP_5' was diluted in water to final concentrations of 5, 10, 20 or 40 ng/μL. Diluted dsRNAs were then applied to 1-week-old wild-type Col-0 seedlings by dipping and spray methods, respectively. Using qRT-PCR, the expression level of dsRNA_EGFP_5' was quantified at 1 day post-treatment (dpt), 2 dpt, 4 dpt or 6 dpt for plants subjected to dsRNA application using the dipping method (**a**) and spray method (**b**), respectively

dsRNA_EGFP_5' and dsRNA_EGFP_3' by the dipping and spray methods. For the dipping method, ten *DR5-EGFP* plants were dipped in 2 mL of sterile water with dsRNA_EGFP_5' or dsRNA_EGFP_3' at a concentration of 20 ng/ μ L (4 μ g per plant). *DR5-EGFP* plants treated with dsRNA_EGFP_5' and dsRNA_EGFP_3' both showed decreased *EGFP*-associated fluorescence from the roots to the elongation zone relative to the control *DR5-EGFP* plants, which were treated with sterile water (Fig. 3a, b). At 6 dpt, *DR5-EGFP* plants treated with dsRNA_EGFP_3' showed fluorescence only in the emerging lateral root caps (Fig. 3b). We also analyzed *EGFP* transcript levels using qRT-PCR, and found that *EGFP* transcript levels were reduced in *DR5-EGFP* plants treated with either dsRNA_EGFP_5' and dsRNA_EGFP_3'. This was true for samples taken at 1, 2, 4, or 6 dpt. All comparisons were made with *DR5-EGFP* plants treated with sterile water. Moreover, at 6 dpt, we found that *EGFP* transcript levels were reduced fourfold in *DR5-EGFP* plants treated with dsRNA_EGFP_5' and reduced tenfold in *DR5-EGFP* plants treated with dsRNA_EGFP_3' compared to control *DR5-EGFP* plants (Fig. 3a, b).

For the spray method, ten *DR5-EGFP* plants were sprayed with 2 mL of sterile water containing dsRNA_EGFP_5' or dsRNA_EGFP_3' at a concentration of 20 ng/ μ L (4 μ g per plant). Fluorescence microscopy was then used to analyze *EGFP*-associated fluorescence in *DR5-EGFP* plants treated with dsRNA_EGFP_5' or dsRNA_EGFP_3'. We found that fluorescence gradually decreased from 1 to 6 dpt (Fig. 4a, b). At 1dpt, fluorescence was similar in *DR5-EGFP* plants treated with dsRNA_EGFP_5', dsRNA_EGFP_3', and sterile water. However, after 2 dpt, fluorescence was lower in *DR5-EGFP* plants treated with dsRNA_EGFP_5' and dsRNA_EGFP_3'. Compared to *DR5-EGFP* plants treated with sterile water, dsRNA_EGFP_5'- and dsRNA_EGFP_3'-treated plants showed gradual decreases in fluorescence in the roots and elongation zone (Fig. 4a, b). At 6 dpt, fluorescence was expressed only in the emerging lateral root caps of *DR5-EGFP* plants treated with dsRNA_EGFP_3' (Fig. 4b). Our qRT-PCR data confirmed these results, showing that the *EGFP* transcript levels of *DR5-EGFP* treated with dsRNA_EGFP_5' and dsRNA_EGFP_3' both gradually reduced after 2 dpt. Moreover, at 6 dpt we found that the *EGFP* transcripts had reduced fourfold in *DR5-EGFP* plants treated with dsRNA_EGFP_5' and fivefold in *DR5-EGFP* plants treated with dsRNA_EGFP_3' relative to control plants (Fig. 4a, b).

We also found exogenous application of dsRNA_EGFP_5' and dsRNA_EGFP_3' dsRNAs, whether by dipping or spray method, were able to effectively target *EGFP* mRNAs. Specifically, our data shows that dsRNAs were effectively delivered from the root cap to the

elongation zone of *DR5-EGFP* plants, where they effectively suppressed the fluorescence caused by *EGFP*, as well as *EGFP* transcription. In addition, we found that dsRNA_EGFP_3', which targets the 3' region of the *EGFP* mRNA, was more effective than dsRNA_EGFP_5', targeting 5' region of *EGFP* mRNA in suppressing *EGFP* expression (Figs. 3 and 4). To be clear, in plants treated with dsRNA_EGFP_5', qRT-PCR was performed using the 3' region of the *EGFP*-specific primer, while for plants treated with dsRNA_EGFP_3', qRT-PCR was performed using the 5' region of the *EGFP*-specific primer. These primers are listed in Additional file 1: Table S4.

Discussion

In this study, we treated a transgenic *DR5-EGFP* line that overexpressed *EGFP* in a Col-0 background with two dsRNAs that specifically targeted *EGFP*. Our results showed that the application of exogenous dsRNAs targeting *EGFP* could suppress fluorescence and *EGFP* transcript levels after 1dpt (Figs. 3 and 4). In addition, despite the high concentration of dsRNA, the dsRNA was not delivered to the plants as much. We found that a 20 ng/ μ L concentration of approximately 500 bp dsRNA was efficiently delivered in 1-week-old *Arabidopsis*.

Interestingly, when plants were treated with dsRNAs targeting *EGFP*, we found that the mRNA transcript levels were higher than in control plants despite the decrease in fluorescence at the initial dsRNA treatment stage. These results may be due to epigenetic modification or other unknown effects caused by siRNAs generated from dsRNAs. Additional studies involving bisulfite sequencing, chromatin immunoprecipitation sequencing, and/or small RNA sequencing may be necessary to elucidate these effects in the future.

The conventional RNAi applications are based on the virus-induced gene silencing, using recombinant viruses which expressed transgenes to produce dsRNA against selected targets (i.e., host-induced gene silencing) in plants [14, 15]. However, as the human population increases, the interest in the nutritional improvement of crops as well as the safety of transgenic plants is also increasing. Therefore, RNAi technology using dsRNA can be used as a useful technique capable of regulating plant properties without genomic manipulation or the use of recombinant viruses or transgenes [1, 6]. However, additional costs associated with the production of dsRNA may be involved in using dsRNA-based RNAi technologies for field-scale management [6]. Our study specifies the concentration, length, and dsRNA-based application methods that can provide an insight into the direct application of dsRNAs to overcome this issue by providing a foundation for RNAi technology using dsRNA.

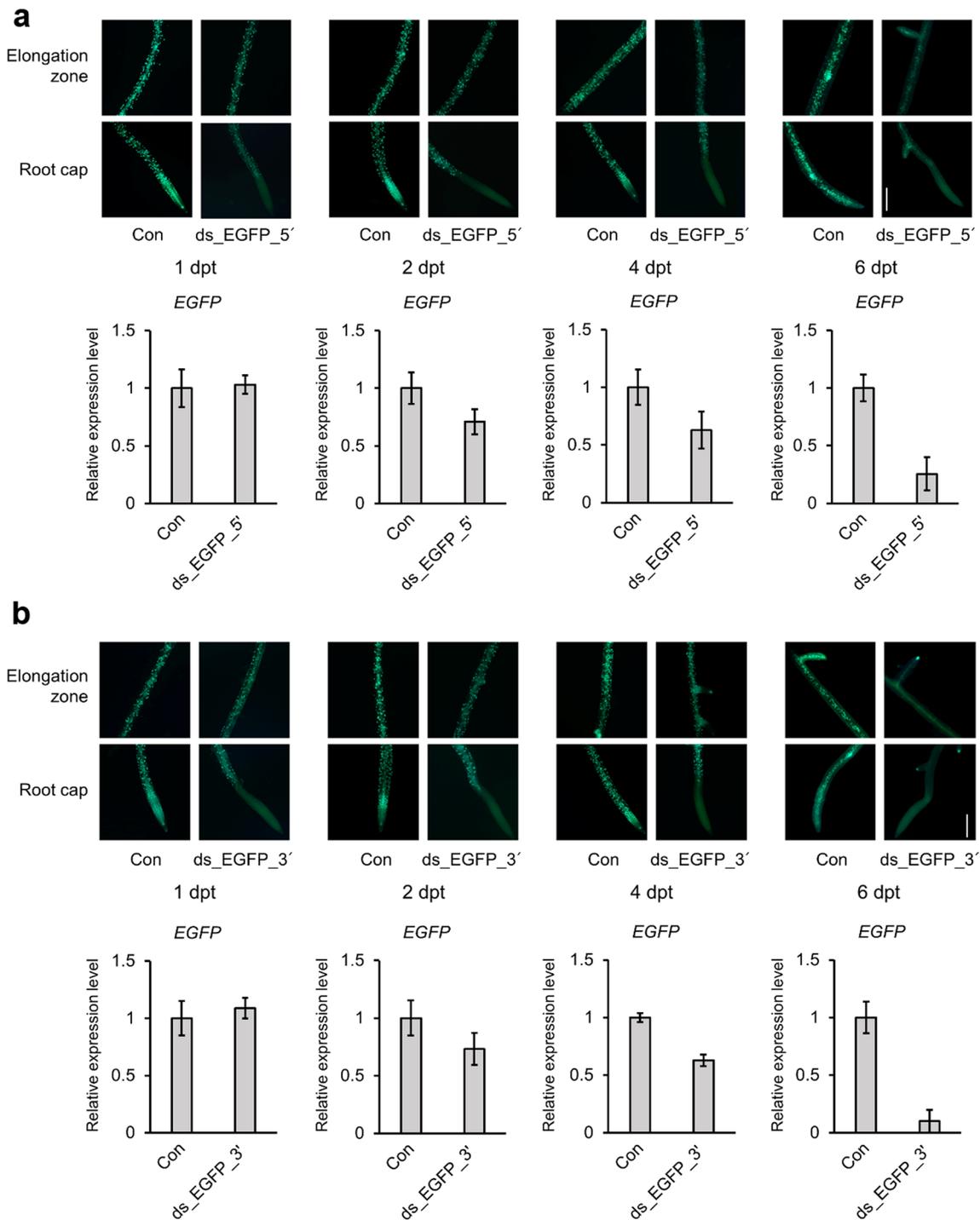


Fig. 3 Analysis of fluorescence and *EGFP* mRNA expression levels in *DR5-EGFP* plants treated with *dsRNA_EGFP_5'* and *dsRNA_EGFP_3'*, respectively, using dipping method. **a, b** Analysis of fluorescence and quantification of *EGFP* mRNA transcript levels of *DR5-EGFP* plants treated with *dsRNA_EGFP_5'* (**a**) and *dsRNA_EGFP_3'* (**b**), respectively, at 1 dpt, 2 dpt, 4 dpt or 6 dpt via dipping. Scale bars = 100 μ m. dpt: Days post-treatment. Con: Treated with sterile water. *ds_EGFP_5'*: Treated with sterile water containing *dsRNA_EGFP_5'*. *ds_EGFP_3'*: Treated with sterile water containing *dsRNA_EGFP_3'*

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s13765-022-00675-0>.

Additional file 1: Figure S1. The scheme of dsRNA synthesis using MEGAscript RNai Kit (Invitrogen, United States). **Figure S2.** The purity check of dsRNA_EGFP using agarose gel electrophoresis. 1: After transcription, 2: After nuclease treatment, 3: After purification, M: 100 bp marker.

Table S3. List of primer sequences used in dsRNA synthesis. **Table S4.** List of primer sequences used in qRT-PCR.

Additional file 2: Table S1. Off-target prediction of dsRNA_EGFP_5' in *Arabidopsis thaliana* using dsCHECK tool.

Additional file 3: Table S2. Off-target prediction of dsRNA_EGFP_3' in *Arabidopsis thaliana* using dsCHECK tool.

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Authors' contributions

CS, GJ and YDC conceived of the research and designed the experiments. GJ and YDC provided plasmids and plants for the experiments. MP and TYU conducted the experiments. CS and MP prepared the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

Not applicable.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

The authors grants the publisher permission to publish the work in *Applied Biological Chemistry*.

Competing interests

The authors declare that they have no competing interests.

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