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SPECIAL TOPICS
Experimental methods and emerging technologies in weed science

RESEARCH ARTICLE

Precise transcript targeting using RNAi for weed control

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Abstract: Background: RNA interference (RNAi) is a natural post-transcriptional gene silencing (PTGS) system of eukaryotic cells and can be used as spray-induced gene silencing (SIGS) to target genes. Due to the complexity of plant cell structure and genome, using SIGS in plants has faced many barriers. **Objective:** We aimed to develop a delivery strategy and appropriate siRNA design to induce PTGS by SIGS. **Methods:** The rice *Phytoene desaturase* (*OsPDS*) gene was used as a model to test our SIGS. Three siRNAs (siPDS1, siPDS2, and siPDS3) targeting different regions of the *OsPDS* transcript were applied individually or mixed siPDS2+siPDS3 in solution with Silwet™ adjuvant. Treatments were applied using a micropipette in rice seedlings (V2-stage), with the addition of proper control treatments: untreated-check and adjuvant-check. Seedlings were accessed phenotypically, physiologically, and molecularly. **Results:** The most prominent phenotype was observed for siPDS1 and siPDS2+siPDS3. Reduction in plant growth, chlorophyll index and shoot dry mass were reduced after siPDS2+siPDS3 application, while increasing anthocyanin content. *OsPDS* was downregulated after siPDS1 application with no change in the non-treated upper leaf. **Conclusions:** Our siRNA design and delivery strategy efficiently delivered the siRNA inside the plant and promoted target PTGS and compatible phenotype. The system used in this experiment did not show systematicity in the plant, probably due to the fast oxidative stress that hindered the systematic effect. This is an advance on the SIGS and can be a valuable tool for PTGS to find new targets for RNAi.

Keywords: Post-transcriptional Gene Silencing; siRNAs; Exogenous Application; Gene Expression

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1. Introduction

RNA interference (RNAi) is an emerging technology with great potential for crop protection (Westwood et al., 2018; Cagliari et al., 2019; Zabala-Pardo et al., 2022). RNAi is a naturally occurring post-transcriptional gene regulation system in eukaryotic cells, and it was first described by Fire et al. (1998) when it was realized that providing a double-stranded RNA led to growth abnormalities in *Caenorhabditis elegans*. The RNAi system is triggered by small-interfering RNA (siRNA) and microRNA (miRNA) molecules that are recognized by the Argonaute protein, which uses a strand as a guide to identify the target messenger RNA (mRNA) (Carthew, Sontheimer, 2009; Wu et al., 2020). The complementary between mRNA and siRNAs induces the RNA-induced silencing complex (RISC) that cleaving the newly formed double-stranded RNA, leading to post-transcriptional gene silencing (PTGS) (Neumeier, Meister, 2021; Iwakawa, Tomari, 2022).

The RNAi system can be used through exogenous applications, known as spray-induced gene silencing (SIGS) (Sammons et al., 2011; Koch et al., 2019). Silencing essential key genes in plants can be a valuable tool for weed management, however, as an emerging technology, it requires improving the RNAi delivery methods in plants. In RNAi studies, *phytoene desaturase* (*PDS*) is a common target gene due to its characteristic phenotype (Sammons et al., 2011; Mellado-Sánchez et al., 2020). The silencing of *PDS* leads to lower levels of carotenoids, reducing photoprotection and increasing oxidative stress, resulting in chlorophyll loss, necrosis, and bleaching (Qin et al., 2007; Park et al., 2017).

Developing a simple methodology to design and apply exogenous RNAi is vital for the development of the SIGS strategy. Thus the use of a plant model like rice enables knowledge acquisition and subsequently facilitates elucidating the process for targeting genes in weeds. Thus, we aimed to develop a delivery strategy and appropriate siRNA design to induce PTGS by SIGS of siRNAs.

2. Material and Methods

We tested the exogenous application of siRNAs in plant cells using rice (*Oryza sativa* L.) as a model plant since we have a vast amount of information related to its genomics and, due to its similarities with weedy rice, our future candidate weed species.

Nipponbare seedlings were germinated in the grown chamber at (day/night) 28/24 °C and 12h photoperiod.

2.1 siRNAs design

The CDS sequence of the *OsPDS* gene (Os03t0184000-01) was accessed in the RAP-DB (https://rapdb.dna.affrc.go.jp/). To search for the most suitable region to place the siRNAs, the CDS sequence was accessed to search for the formation of secondary structures using the RNAfold web server (http://rna.tbi.univie.ac.at/cgibin/RNAWebSuite/RNAfold.cgi) and RNAstructure (https://rna.urmc.rochester.edu/RNAstructureWeb/Servers/Predict1/Predict1.html). Non-folding regions were selected for siRNA design. Three 21 bp siRNAs (siPDS1, siPDS2, and siPDS3) were designed to target different regions of the *OsPDS* CDS (Elbashir et al., 2001). The targeting region of each siRNA in the *OsPDS* transcript is shown in Figure 1. To minimize off-targeting effects, the specificity of the siRNAs was analyzed in silico using the BlastN tool available at NCBI (https://www.ncbi.nlm.nih.gov/) and rice genome (*Oryza sativa* L. *japonica*) as reference. Only specific sequences were utilized for siRNA synthesis. The double-stranded siRNAs were designed with two nucleotides in overhang at the 3' end (Elbashir et al., 2001). The siRNAs were modified at 5' end of the strands with a phosphate group that is decisive for the association of the siRNA with the RISC complex (Schwarz et al., 2002) and with a 2'-*O*-methylation in the 3' end of ribose as it increases the stability of the siRNA molecule (Ji, Chen, 2012) (Table 1). The siRNAs were in vitro synthesized by Sigma-Aldrich® company with HPLC grade purification.

2.2 Delivery strategy

Before running this experiment, a pre-experiment was performed to determine the best delivery system for the RNAi using a homemade dsRNA (long double-stranded RNA) since it is the most difficult strategy to reach specifically a target gene due to its random cleavage by the RISC. The treatments tested were application via spray, micropipette (both applied with and without leaf injury caused by sandpaper), application at the abaxial side, microneedle, and root absorption. Application with a micropipette without leaf injury was our best result, with a suitable phenotype and high precision of RNAi concentration reaching the leaf (data not shown). A pre-experiment was also performed to determine the Silwet™ (organosiliconized, Rizobacter®) concentration, testing Silwet™ 0.01%, 0.08%, and 0.10% v/v in volumes of 1, 2, 3, and 5 µL. Silwet™, 0.08% in 5 µL, was the higher concentration applied that did not cause any phenotypic changes and provided optimal liquid distribution on the leaf surface (data not shown).

The experiment was conducted in a completely randomized design with four replications. The treatments were applied individually, siPDS1, siPDS2, or siPDS3, and in a mixture of siPDS2+siPDS3 and controls (untreated check and adjuvant check). Solutions containing 1 µg/µL of siRNA were prepared with the addition of 0.08% v/v of Silwet adjuvant, while the combination siPDS2+siPDS3 contained 2 µg/µL. The application was performed using a micropipette, depositing 5 µL (5 droplets of 1 µL) of solution in the first expanded leaf on the adaxial surface in rice seedlings at the V2 stage. After application, plants were kept in a growth chamber (day/night 28/24 °C temperature, 12 h photoperiod, and light intensity of 300 µmol/m^2/s) until the end of the experiment.

2.3 Phenotyping

Plants were analyzed daily to identify phenotypic changes, and the advancing effects were recorded as photos. Two chlorophyll index and anthocyanin readings were taken per plant at 7 and 14 days after application (DAA) using a Dualex Force-A leaf analyzer model. The shoot height of rice plants was measured at 7 and 14 DAA using a ruler. Shoots were collected at 14 DAA to quantify shoot dry weight. Shoots were kept in paper bags and dried in an air force oven until samples reached a stable weight. Four replicates were taken per treatment.

2.4 Gene expression analysis

Leaf tissues were collected from three replicates for treatment 48h after application (HAA). Total RNA was

Table 1 - siRNAs and its modification used to target rice Phytoene desaturase

siRNA	5' modification	siRNA sequence	3' modification
siPDS1	Phosphate	UUGGUAUGAAACUGGGCUUCA	2'-*O*-methyl
		AAGCCCAGUUUCAUACCAAUC	
siPDS2	Phosphate	GAGAAUUCAGCCGGUUUGAUU	2'-*O*-methyl
		UCAAACCGGCUGAAUUCUCCU	
siPDS3	Phosphate	UAUGGGCCAUACUAAGAAACA	2'-*O*-methyl
		UUUCUUAGUAUGGCCCAUAUU	

Underlined nucleotides represent the 2 overhang nucleotides of each strand, passenger and guide

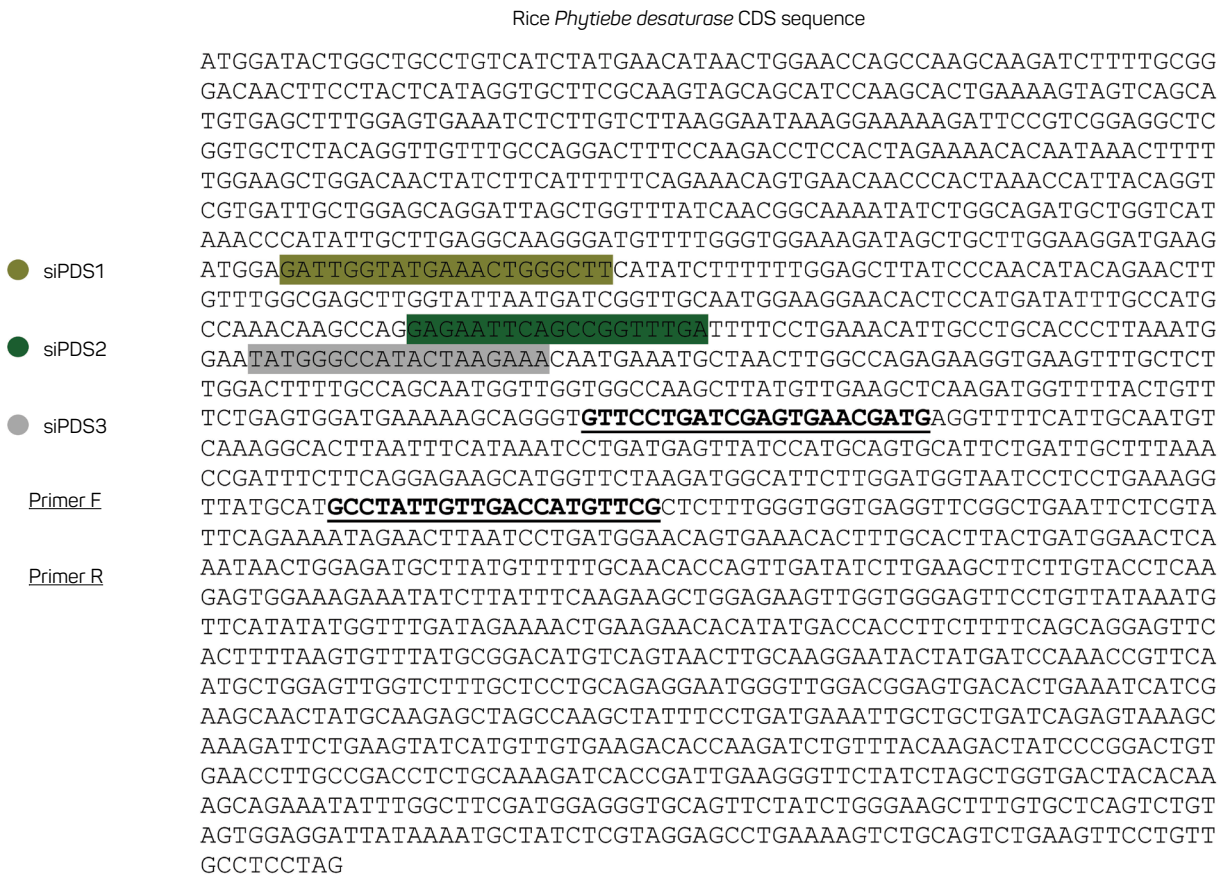

Figure 1 - Schematic representation of the CDS sequence of the gene coding for the rice Phytoene desaturase (OsPDS) highlighting the targeting region of the three siRNAs (siPDS1, siPDS2, and siPDS3) and the primers used for the *OsPDS* gene expression quantification in RT-qPCR

extracted from the leaf that received treatment (untreated-check, adjuvant-check, and siPDS1) independently from each plant and from the upper leaf (to test siRNA mobility) using Pure Link Plant RNA Reagent (Invitrogen®), following manufacturer recommendations. The quality and integrity of each RNA was accessed using a spectrophotometer and agarose gel electrophoresis, respectively. The cDNA synthesis was performed using Super Script First Strand Synthesis Kit (Invitrogen®) and oligo(dT), following manufacturer recommendations.

Gene expression was quantified by RT-qPCR according to the MIQE Guidelines (Bustin et al., 2009) using oligonucleotides for the target and reference genes (Table 2) and three technical replicates in a Lightcycler 96 well plate and foil (Roche) in a CFX Opus thermocycler (Bio-rad). Validation tests were performed to determine each oligonucleotide's amplification efficiency and specificity using four cDNA dilutions. The selected oligonucleotides showed efficiency between 90 and 110% and a single peak in the dissociation curve. Reactions were performed using 1 µL of cDNA at a 1:25 dilution (determined in the validation experiment), 11.0 µL of UltraPure™ DNase/RNase-Free Distilled Water (Invitrogen®), 0.25 µL of ROX Reference Dye (Invitrogen®), 2.0 µL 10× PCR Buffer (Invitrogen®), 1.5 µL 50 mM Magnesium Chloride (Invitrogen®), 0.05 µL Platinum' Taq DNA Polymerase, 0.2 µL dNTPs, 3.0 µL SYBR Green I (Invitrogen®), and 1.5 µL of each oligonucleotide (forward and reverse) in a reaction of 20 µL final volume. Reactions without cDNA were also performed for each pair of oligonucleotides and used as a negative control. PCR reactions were performed in the following conditions: initial denaturation at 95 °C for 5 min; 40 cycles of 95 °C for 20 s, 60 °C for 15 s, and 72 °C for 20 s. Conditions for the melting curve were: 95 °C for 5 s, 70 °C for 1 min, and a temperature ramp up to 95 °C, increasing by 0.5 °C each 5 s.

OsPDS expression was quantified using the delta-delta CT method (Livak, Schmittgen, 2001), and the expression of the *OsPDS* from the untreated check as the baseline. Normalization was performed with three reference genes: *OsEF1α*, *OsEF4α*, and *OsGAPDH*. By using the delta-delta CT method, values of the relative quantification (RQ) in the control condition (baseline) is fixed in 1.0, so that the RQ values of the target genes in the treatments are interpreted as: positive values from 0.0 to 1.0 are considered downregulated

and positive values above 1.0 are considered upregulated. To be more intuitively interpreted, we converted RQ values in Log^{10} being considered downregulated the genes having negative values and upregulated the genes having positive values. Graphically, gene expression was represented as Log^{10} in violin bar, showing the median, and the quartiles using the Prism GraphPad Software (Version 10.2.3 (347), April 21, 2024).

3. Results and Discussion

3.1 Phenotypic effects of siRNA application

We tested three siRNAs that were applied using a micropipette to target different *OsPDS* regions (siPDS1, siPDS2, and siPDS3). We detected phenotypic changes in the leaves that received siRNAs, but there were no phenotypic changes in the untreated and Silwet at 48 HAA. The upper leaves did not exhibit any visible changes in their phenotype, unlike the treated leaves which showed a necrotic profile. siPDS1 showed the strongest phenotypic effect compared with siPDS2 and siPDS3 (Figure 2). The leaf that received siPDS1 has completely died 72 HAA, showing a necrotic profile probably due to the oxidative stress caused by *OsPDS* silencing (Kruse et al., 2001; Park et al., 2017). Functional genomic studies generally analyze *PDS* in different plant species, such as *Arabidopsis thaliana*; the primary phenotype is related to albino leaves caused by photo-bleaching (Qin et al., 2007; Vaia et al., 2022); however, our findings are consistent with the effect of the synergism between carotenoid synthesis and photosystem II inhibiting herbicides that cause oxidative stress in plants since carotenoids act as a quenching of reactive oxygen species in the chloroplast and can alleviate the oxidative stress caused by the electron transport in the form of heat (Kruse et al., 2001; Park et al., 2017).

Considering that siPDS2 and siPDS3 caused minor phenotypic changes compared with siPDS1, we mixed both siPDS2 and siPDS3 to check if they can promote an

Table 2 - Oligonucleotides used in RT-qPCR analysis to quantify *OsPDS* gene expression

Gene	Sequence (5′ -3′)	Reference
Phytoene desaturase (*OsPDS*)	F – GTTCCTGATCGAGTGAACGATG R – CGAACATGGTCAACAATAGGC	Andrieu et al. (2012)
Elongation factor 1α (*OsEF1α*)	F – TTTCACTCTTGGTGTGAAGCAGAT R – GACTTCCTTCACGATTTCATCGTAA	Zhou et al. (2012)
Elongation factor 4α (*OsEF4α*)	F – TTGTGCTGGATGAAGCTGATG R – GGAAGGAGCTGGAAGATATCATAGA	Jain et al. (2006)
Glyceraldehyde-3-phosphate dehydrogenase (*OsGAPDH*)	F – AAGCCAGCATCCTATGATCAGATT R – CGTAACCCAGAATACCCTTGAGTTT	Jain et al. (2006)

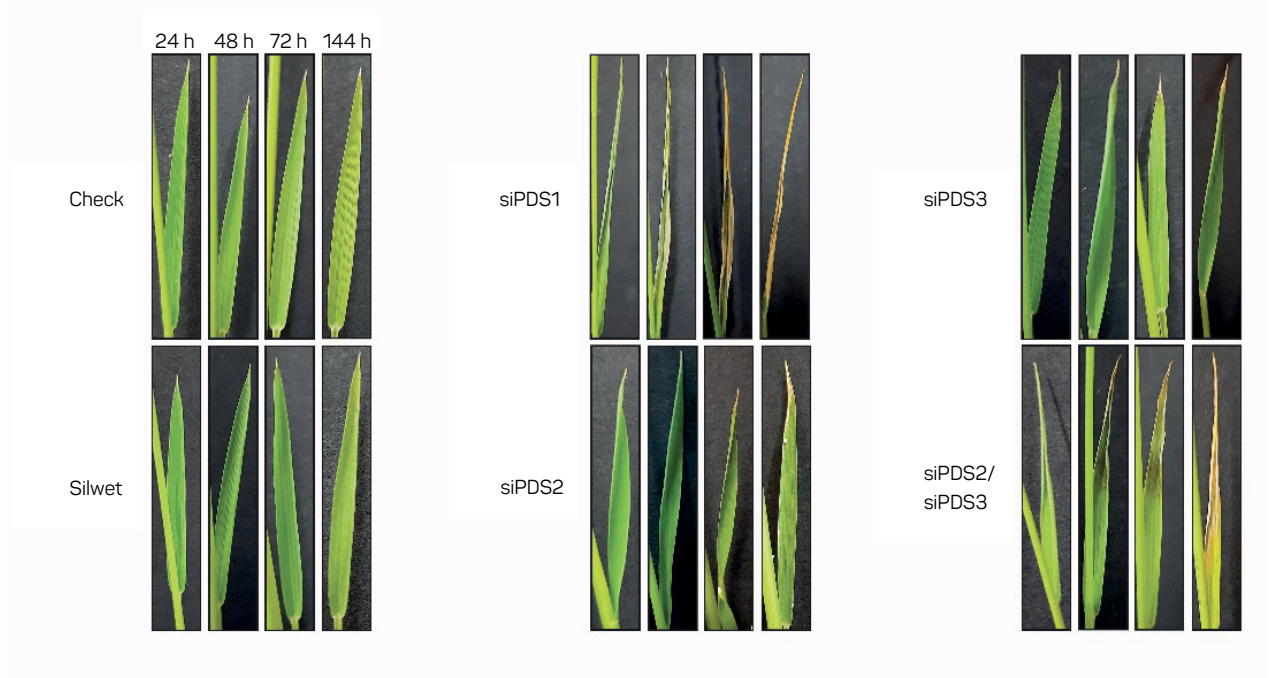

Figure 2 - Phenotypic effects in V2 stage rice seedlings along the time after treatment with Silwet, Silwet + siRNAs (siPDS1, siPDS2, siPDS3 and mixture of siPDS2 + siPDS3) and untreated check

increased phenotypic effect. siPDS2+siPDS3 leads to a most prominent phenotypic effect when compared with the separate application. The observed effect took longer to develop than siPDS1, but we found a similar profile of oxidative stress and leaf death beginning at 144 HAA (Figure 2). Overall, the targeting site of siRNA1 seems promising when looking for a phenotypic change caused by *OsPDS* targeting.

3.2 Physiological effects of siRNA in rice plants

Due to the phenotypic effect of siRNA application that causes leaf necrosis possible due to oxidative stress, the effect on chlorophyll index at 7 and 14 DAA was evaluated. The application of the mixture of siPDS2+siPDS3 resulted in a reduction in the chlorophyll index at 7 DAA, but the same effect was not observed by the application of siPDS2 or siPDS3 (Figure 3A). At 14 DAA, no difference in chlorophyll index was detected (Figure 3A). The reduction in chlorophyll index in plants that received siRNAs targeting *OsPDS* may be an effect of the reduction in the cellular pool of carotenoids that increases the susceptibility of chlorophyll to oxidative stress, as these pigments serve as protectors for the photosystem (Qin et al., 2007; Park et al., 2017).

Anthocyanins were analyzed since they indicate plant stress and play an essential role in oxidative stress in scavenging reactive oxygen species (Davison et al., 2002; Li, Ahammed, 2023). Higher anthocyanin content was detected in 7 DAA in leaves treated with siPDS2 + siPDS3 compared to leaves treated with siPDS2, siPDS3, and the untreated and adjuvant checks (Figure 3B). It corroborates with the findings of the chlorophyll index at 7 DAA and with the symptoms of oxidative stress in leaves that received siPDS2 + siPDS3.

To quantify the effect of siRNA on plant development, we accessed plant height and shoot dry weight (SDW).

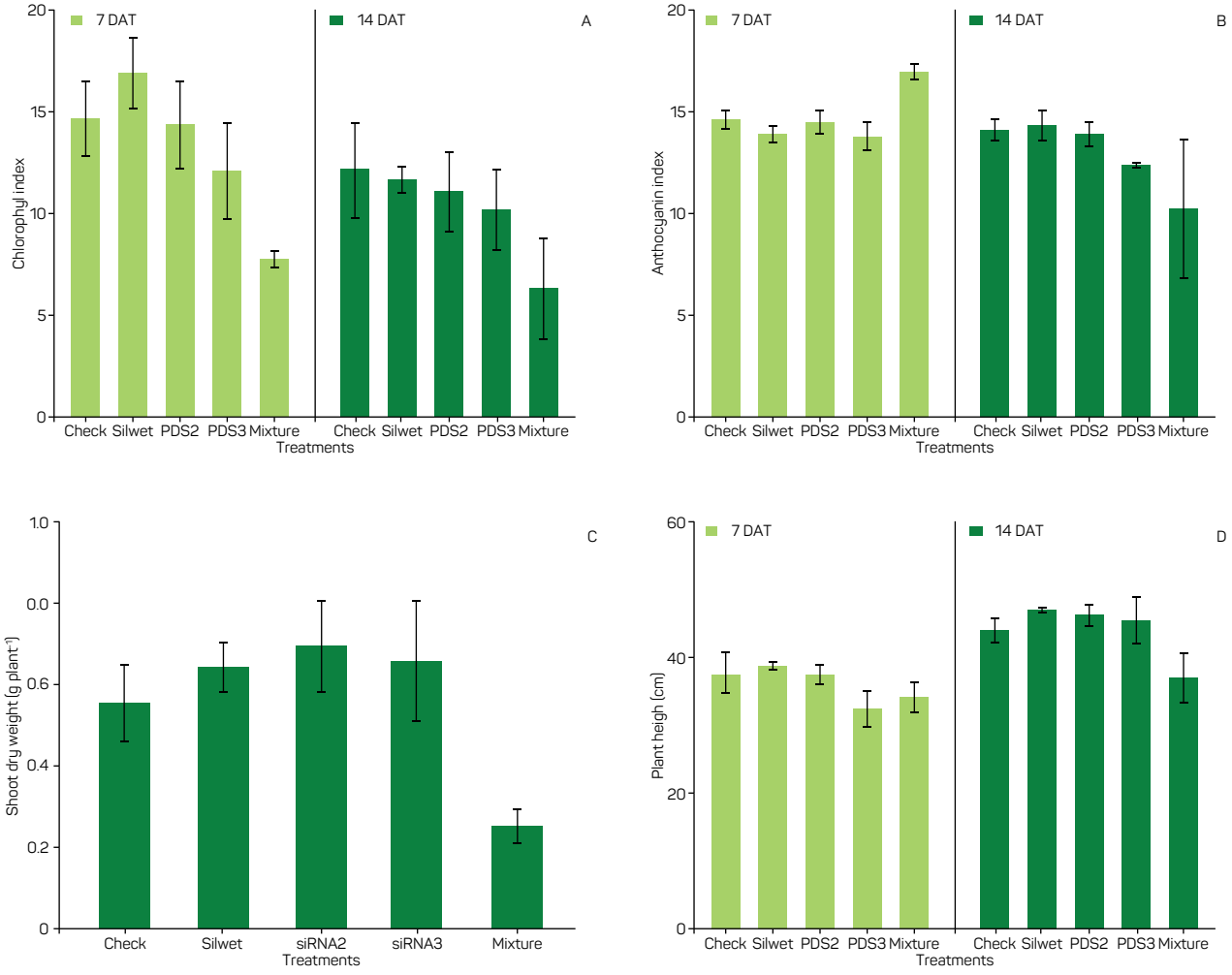

Figure 3 - Effect of the application of different siRNA treatments (siPDS2, siPDS3 and a mixture of siPDS2+siPDS3) on rice plants (A) chlorophyll index at 7 and 14 days after treatment application, (B) anthocyanin index at 7 and 14 days after treatment application, (C) shoot dry weight at 14 days after application of treatments, and (D) plant height at 7 and 14 days after of treatments. The check received only water; Silwet was used as a check to observe its effect alone, and all the other treatments received Silwet at 0.08%. Error bars correspond to a 95% confidence interval (n = 4)

No effect on SDW was observed in plants that received siPDS2 or siPDS3 (Figure 3C). The height of the plants in 7 DAA did not show any significant difference; however, in 14 DAA, siPDS2 + siPDS3 led to less plant growth, resulting in a height of 37.75 cm. SDW was negatively reduced by the treatment of the mixture of siPDS2 + siPDS3, showing a 42.5% reduction compared to the untreated check and adjuvant (Figure 3D). Unfortunately, we did not have plant material from siPDS1 treatment to do this analysis, so they were not shown. Our findings are compatible with functional genomics studies showing that disruption of *PDS* in *A. thaliana* causes a dwarf phenotype that was associated with the PDS pool that can be a crucial factor affecting carotenoid, chlorophyll, and gibberellin biosynthesis pathways (Qin et al., 2007; Park et al., 2017).

3.3 Transcriptional gene silencing induced by siRNA

To check for the PTGS of the *OsPDS* upon siRNA application, we quantified the *OsPDS* transcript amount by RT-qPCR in the siPDS1-treated leaves 48 HAA due to the strong phenotype. The specific place of the primers used for *OsPDS* is shown in Figure 1. We detected a significant downregulation of *OsPDS* in siPDS1-treated leaves compared with adjuvant-check leaves (Figure 4). The downregulation of *OsPDS* suggests that our delivery method and the siRNA design efficiently target *OsPDS*, causing PTGS.

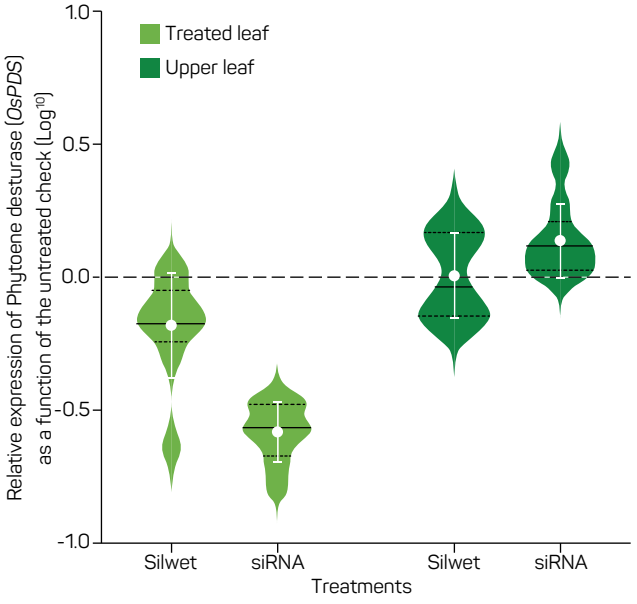

Figure 4 - Relative expression of *Phytoene desaturase* (*OsPDS*) as a function of the untreated check (Log^{10}) in the treated and the upper leaf as affected by the application of siRNA1+Silwet and Silwet alone (secondary check to observe its effect alone), 48 hours after application. In each violin bar, the black-solid line represents the median, and the two dotted lines represent the quartiles. The white circle represents the mean, and the white error bars represent the 95% confidence intervals (n = 9)

The siRNA applied to the plants resulted in reduced chlorophyll index, height, and SDW. In front these physiological changes in the plants, we analyzed the transcript levels of *OsPDS* in the leaf above the siPDS1-treated leaf to determine if siRNA treatment is systemic. No difference in transcript accumulation was detected in siPDS1, adjuvant-check, and untreated-check (Figure 4). We could not find changes in the *OsPDS* transcript amount in the upper leaf, probably due to the fast action of the RNAi system observed in siPDS1-treated plants. In plants, the local stress sensing and signaling can trigger a systemic response to acclimate itself and it seems the case of siRNA-treated plants that showed morphological and physiological changes after application (Zandalinas et al., 2020).

3.4 Prospective aspects of siRNA application in plants

We used a conceptual target with an easy phenotype and a model crop plant showing a complete and curated genome to minimize unknown off-targets strategically to test our delivery system and siRNA design. As we could detect phenotypic, physiological, and molecular changes compatible with the *OsPDS* silencing considering functional genomics studies, it can be extrapolated to target other genes of interest for weed control. As we used a diploid and monocot plant, our system should be tested for other plant species (crops and weeds).

In addition, new tests involving concentration, mixture, and delivery strategies should be performed to improve the delivery of siRNAs, maximize the cell effects, and reduce the costs of siRNA synthesis, a vital issue underlining siRNA experiments. Overall, our findings can be used as a base to develop new studies using siRNAs in plants and to improve RNAi technology even as a functional genomics tool since it is a transient PTGS or even for application in weed science targeting essential genes in weeds.

4. Conclusions

Our approach of using siRNA molecules targeting *OsPDS* was effective, as it caused phenotypic and transcriptional changes. Of the three siRNA molecules tested, siPDS1 had a stronger visual impact than siPDS2 and siPDS3. However, when siPDS2 and siPDS3 were combined, and their concentration was increased, they caused a more significant phenotypic effect than when used separately. We could not detect transcriptional changes in another leaf than the siRNA treated. The system used in this experiment did not show systematicity in the plant, probably due to the fast action of the treatments used and the fast oxidative stress that hindered the systematic effect; on the other hand, it caused growth arrest, which has been associated with the regulation of other biosynthetic pathways. This is an advance on the SIGS and can be a useful tool for PTGS and to find new targets for RNAi.

Author's contributions

All authors read and agreed to the published version of the manuscript.

LAA, VEV, ERC, and RAP: conceptualization of the manuscript and development of the methodology. VEV and RAP: data collection and curation. LAA, VEV, and RAP: data analysis. LAA, ERC, VEV, and RAP: data interpretation. LAA, ERC, and VEV: funding acquisition and resources. LAA and ERC: project administration. LAA and ERC: supervision. LAA, VEV, and RAP: writing the original draft of the manuscript. LAA, ERC, VEV, and RAP: writing, review, and editing.

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