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Efficient virus-induced gene silencing in *Cynodon dactylon* and *Zoysia japonica* using rice tungro bacilliform virus vectors

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ABSTRACT

Cynodon dactylon and *Zoysia japonica* are two important warm-season turfgrass species with multiple utilizations. Genetic transformations of *Cynodon dactylon* and *Zoysia japonica* are extremely difficult, which severely restrict the gene functional studies and molecular breeding research of the two grasses. By contrast, virus-induced gene silencing (VIGS) presents an effective and rapid alternative method to create targeted gene knock-outs for gene functional studies in plants. In this study, we tested the efficiency of modified rice tungro bacilliform virus (RTBV) vector in silencing *Cynodon dactylon* and *Zoysia japonica* phytoene desaturase (*PDS*) gene through agroinfiltration. The agroinfiltrated leaves of two *Cynodon dactylon* and two *Zoysia japonica* cultivars all showed white streaks typical for *PDS* gene silencing at 21 days post agroinfiltration. In *Cynodon dactylon* and *Zoysia japonica* plants displaying white streak leaf symptoms, *PDS* gene expression in leaves were all significantly lower than that of control and mock-infected plants. These results indicated that RTBV-VIGS system is efficient in *Cynodon dactylon* and *Zoysia japonica*.

1. Introduction

Perennial grasses *Cynodon dactylon* and *Zoysia japonica* are two important warm-season turfgrass species. Because of their superior morphological characteristic and turf performance, *Cynodon dactylon* and *Zoysia japonica* are widely used in home lawns, public parks, golf courses, sport fields and other purpose (Patton et al., 2004). In addition to their great economical value, *Cynodon dactylon* and *Zoysia japonica*, along with other turfgrass species, are also good plant materials to explore growth regulation and stress response mechanisms of perennial monocot plants (Huang et al., 2014). To facilitate the molecule breeding research and gene functional studies, both biolistic and *Agrobacterium*-mediated genetic transformation of *Cynodon dactylon* and *Zoysia japonica* were successfully developed (Zhang et al., 2003; Li et al., 2005; Ge et al., 2006), however, the inefficient and laborious transformation procedures impeded their extensive application.

Virus-induced gene silencing (VIGS) is an effective technology that employs recombinant viruses to specifically down-regulate endogenous gene expression through plant innate silencing mechanisms called post-transcriptional gene silencing (PTGS) (Purkayastha and Dasgupta, 2009; Becker and Lange, 2010). Since its first application in silencing of phytoene desaturase (*PDS*) gene in *Nicotiana benthamiana* (Kumagai et al., 1995), VIGS experiments have been successfully performed in many eudicot plants including *Arabidopsis thaliana*, *Glycine max*, *Gossypium hirsutum*, *Lycium barbarum* and *Prunus avium* (Burch-Smith et al., 2006; Nagamatsu et al., 2007; Qu et al., 2012; Liu et al., 2014; Kawai et al., 2016). In comparison to the large number of VIGS vector systems adapted for eudicot plants, only few VIGS systems have been established for monocots. The two RNA viruses *barley stripe mosaic virus* (BSMV) and *brome mosaic virus* (BMV) were adopted for VIGS in monocot plants including *Hordeum vulgare*, *Brachypodium distachyon*, *Oryza sativa* and *Zea mays* (Ding et al., 2006; Pacak et al., 2010), however, the VIGS procedures using these two RNA viruses are inconvenient mainly because of the requirement of complicated *in vitro* RNA transcription. By contrast, VIGS using the DNA viruses, including *rice tungro bacilliform virus* (RTBV), through agroinoculation are more convenient and efficient (Purkayastha et al., 2010; Purkayastha et al., 2013).

In this study, we tested the feasibility of RTBV-VIGS system in the two warm-season turfgrasses *Cynodon dactylon* and *Zoysia japonica* using *PDS* gene as a reporter. The agroinfiltrated leaves of two *Cynodon dactylon* and two *Zoysia japonica* cultivars all showed

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Table 1
Primers used in this study.

Primer Name	Primer Sequence (5′-3′)
PDS-F-290	RCAGYRAACRMCCCASTAAR
PDS-R-980	AAATCKGTTYARAGCAATCARAAT
3′-PDS-1	TGTCCAAGGCACTCAATTTCA
3′-PDS-2	CTGATGAGTTATCAATGCAGT
5′-PDS-1	CATCTCTTGCTTCAAGCAGTA
5′-PDS-2	TATGGCCAGCATCTGCCAAAT
adaptor-1	CCAGTGAGCAGAGTGACG
adaptor-2	GAGGACTCGAGCTCAAGC
adaptor-oligodT	CCAGTGAGCAGAGTGACGAGGACTCGAGCTCAAGCTTTTTTTTTTTTTTTT
PDS-VIGS-F	ACGCGTACAGAATTTGTTTGGCGAGCT
PDS-VIGS-R	TTAATTAAGAACCATGCTTCTCCTGAAGA
CdPDS-q-F	TGTCTTTACGGCACCAGTTGAT
CdPDS-q-R	GGCCAGCTCTTCCATAGTTGC
CdACT1-q-F	GCTCAACCCCAAGGCTAAC
CdACT1-q-R	AGAGCGTATCCCTCGTAGATG
ZjPDS-q-F	CTGCCGTGATTTCCCAAGAC
ZjPDS-q-R	ATACCAAGCTCGCCAAACAAAT
ZjACT1-q-F	GGCAACATTGTGCTCAGCGGTGG
ZjACT1-q-R	AACCACCTTAATTTTCATGCTGC

Underline indicate the recognition sites of restriction endonuclease *Mlu*I and *Pac*I.

white streaks typical for *PDS* silencing at 21 days post infection (DPI), meanwhile the expression level of *PDS* gene in leaves with white streak symptoms were all significantly lower than that of control and mock-infected plant leaves, clearly indicating that RTBV-VIGS system is efficient in *Cynodon dactylon* and *Zoysia japonica*. To our knowledge, this is the first report of VIGS in warm-season turfgrasses.

2. Materials and methods

2.1. Plant materials and growth condition

The third stolon nodes of *Cynodon dactylon* 'Yangjiang', 'Common' and *Zoysia japonica* 'Lanying No.3', 'Crowne' were cut from the original plants growing in the experimental field of Nanjing Botanical Garden, fixed in a floating foam board and transferred to the Hoagland's nutrient solution as described (Chen et al., 2014). The node-originated new grass seedlings were grown in a growth chamber at 28 °C with a photoperiod of 16 h/8 h of white light.

2.2. PDS *gene cloning and sequence analysis*

Total RNA was extracted from the mature leaves of *Cynodon dactylon* 'Yangjiang' and *Zoysia japonica* 'Crowne' using Plant RNA Kit (Omega, GA, USA). The first strand cDNA was synthesized using the SuperScript II reverse transcriptase kit (Takara, Dalian, China). Based on the conserved domain of PDS proteins in other species, a pair of degenerated primers PDS-F-290 and PDS-R-980 (Table 1) was designed to amplify partial fragments of *CdPDS* and *ZjPDS* genes using the synthesized cDNA as template. For 3′ RACE reaction, the first-strand cDNA was synthesized using an adaptor-oligodT primer, followed by a nested PCR using the primer pair 3′-PDS-1/adaptor-1 and 3′-PDS-2/adaptor-2 (Table 1). For 5′ RACE reaction, the first-strand cDNA was synthesized using gene-specific primer 5′-PDS-1, followed by an addition of polyA tail using terminal deoxynucleotidyl transferase (Takara, Dalian, China), a one-round PCR using the primer pair adaptor-oligodT/5′-PDS-1 and a nested PCR using the primer pair 5′-PDS-1/adaptor-1 and 5′-PDS-2/adaptor-2 (Table 1). The sequenced 5′ RACE fragment, cDNA partial fragment and 3′ RACE fragment were combined to obtain the full-length cDNA sequence of *CdPDS* and *ZjPDS* genes. The deduced protein sequences were analyzed by ClustalX and the phylogenetic tree was drawn using MEGA 5.05 with the Neighbor-Joining (NJ) method and 1000 bootstrap replicates.

2.3. VIGS vector construction

A 420-bp fragment corresponding to bases 456-875 of *CdPDS* and *ZjPDS* genes were PCR amplified from *Cynodon dactylon* 'Yangjiang' and *Zoysia japonica* 'Crowne' cDNA using *Pfu* DNA polymerase (Transgen, Beijing, China) with primer pair PDS-VIGS-F/PDS-VIGS-R (Table 1). The PCR products were cleaved with *Mlu*I and *Pac*I restriction endonucleases (NEB, MA, USA) and cloned into *Mlu*I-*Pac*I-cut pRTBV-MVIGS vector to generate pRTBV-MVIGS-*CdPDS* and pRTBV-MVIGS-*ZjPDS*, respectively.

2.4. Agroinfiltration

For agroinfiltration, the pRTBV-MVIGS, pRTBV-MVIGS-*CdPDS* and pRTBV-MVIGS-*ZjPDS* vectors were firstly transformed into *Agrobacterium tumefaciens* strain EHA105. PCR-confirmed single colonies were then selected and inoculated in 5 ml of Luria-Bertani medium containing 2 kinds of antibiotics (30 mg/L rifampicin and 50 mg/L kanamycin) and grown overnight in a 28 °C shaker. These overnight starter cultures were subsequently used to inoculate 50 ml cultures and grown overnight at 28 °C. Agrobacterium cultures were harvested by centrifugation at 4000*g* for 15 min and pellets resuspended in an infiltration buffer (10 mM MES, 10 mM $MgCl_2$, and 200 μM acetosyringone, pH 5.6) at an optical density of 2.0 at 600 nm. The resuspended infiltration solution were then incubated at room temperature for 3 h. 7-days-old *Cynodon dactylon* and *Zoysia japonica* seedlings were taken from the Hoagland's nutrient solution, washed gently and immersed in the prepared infiltration solution in beakers. Beakers were placed into a Vacufuge Plus vacuum concentrator (Eppendorf, Hamburg, Germany) and vaccuum applied for 5 min. The vacuum-infiltrated *Cynodon dactylon* and *Zoysia japonica* seedlings were put back to the floating foam board immersed in Hoagland's nutrient solution and were maintained at 28 °C under conditions as described above.

2.5. Quantitative RT-PCR

To determine relative levels of the endogenous *CdPDS* and *ZjPDS* transcripts in infiltrated leaves exhibiting visible silencing phenotypes, quantitative RT-PCR were performed using primer pairs CdPDS-q-F/CdPDS-q-R and ZjPDS-q-F/ZjPDS-q-R (Table 1). These two pairs of primers were designed to exclude the region of cDNA cloned into the pRTBV-MVIGS vector to ensure that only the endogenous mRNA was amplified. For each experiments, leaves