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Prediction of VIGS efficiency by the *S*fold program and its reliability analysis in *Gossypium hirsutum*

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Abstract Genetic transformation in some plant species, including cotton (*Gossypium hirsutum*), is hampered by laborious and time-consuming processes and often unachievable. Virus-induced gene silencing (VIGS) by double-stranded RNAs can serve as a reverse-genetics tool to determine gene function. However, knockdown levels vary greatly when using a tobacco rattle virus-based vector that carries different cDNA fragments of a gene. How to choose the optional target fragment for high interference efficiency is very challenging. Addressing this challenge requires increasing the efficacy of small interference RNA (siRNA) in target fragment. Here, we describe a method to assess VIGS efficiency by comparing the following parameters of siRNA in target sequence: the disruption energy of the target ($\Delta G_{disruption}$), the differential stability of siRNA duplex ends (DSSE), and the internal stability at positions 9–14 of the siRNA antisense strand (AIS), which are calculated by *S*fold program (http://sfold.wadsworth.org). We find that the siRNAs with low $\Delta G_{disruption}$, high DSSE and high AIS have high activity and easily result in high VIGS efficiency by experimentally testing the actual knockdown levels of the four target genes, *GhPDS*, *GhCLA1*, *GhAOS1*, and *GhCXE1* via choosing different target sequences for each gene. Therefore, the *S*fold program can be used to analyze target sequences when carrying out VIGS design to increase gene-silencing effects in plants.

Keywords VIGS · *S*fold program · siRNA · Target sequences · *Gossypium hirsutum*

Xiaoyang Ge, Jie Wu and Chaojun Zhang contributed equally to this work.

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1 Introduction

Homologous gene transformation systems often cosuppress plant gene expression [1]. Furthermore, viral infection can repress expression of plant genes when plant and virus genes share significant homology. Based on homology, a model for plant-mediated RNA degradation as a defense mechanism has emerged [2, 3]. Some RNA and DNA viruses, such as potato virus X, tobacco mosaic virus, tobacco rattle virus, tomato golden mosaic virus, and cabbage leaf curl virus have been successfully used for gene silencing in plants [4–6].

As a defense system, VIGS involves posttranscriptional gene silencing against intracellular viral proliferation and extracellular viral movement [7], and it shares a similar mechanism to the RNAi pathway, referred to as interfering RNA from dsRNA [8]. In general, genetic transformation is

indispensable for confirmation of genes function using the RNAi method. However, few plant species are able to obtain transgenic plants, and plant species transformation is laborious and time-consuming, and often not achievable. Compared with the RNAi method, VIGS is a fast and convenient method for generating phenotypes, and can be used to characterize gene function in a wide range of angiosperm species. Thus, it is widely used for silencing individual gene and whole gene family. Plants with different silencing levels are caused by the diversity of infection patterns, transmission vectors, and plant defenses [9]. For example, TRV is the widely used and effective virus vector in *G. hirsutum* [10]. However, knockdown levels of the target gene and the resultant corresponding phenotypes vary greatly among different target sequences/virus vector combinations [11, 12]. Thus, it is interesting to determine VIGS efficiency in advance.

The secondary structure of target sequence and the activity of siRNA were the two determinants of RNAi efficiency, and it has been supported by both computational modeling and experimental results [13–17]. VIGS by double-stranded RNA molecules acts in the same way as RNAi, being recognized by DICER-like enzymes that cleave dsRNA into siRNA, thus resulting in activation of RNA-induced silencing complex (RISC),which guides recognition and targeted cleavage of homologous target mRNA with the guide strand of siRNA [18, 19]. Based on the similar mechanisms of RNAi and VIGS, we believe that the target sequence also affects VIGS efficiency. To confirm this speculation, the *S*fold program, a software for statistical folding of nucleic acids and studies of regulatory RNAs, was used to predict probable RNA secondary structures [20]. The *S*irna module of the *S*fold software was used to analyze target accessibility and RNA duplex thermodynamics for rational siRNA design, and three main parameters were selected including $\Delta G_{disruption}$, DSSE, and AIS, which were closely related with target accessibility, assembly ability of RISC, and target cleavage ability, respectively. Different target sequences were selected to compare gene knockdown levels, and the VIGS results revealed the *S*fold program was an available tool. Therefore, integrating $\Delta G_{disruption}$, DSSE, and AIS of target sequences facilitates obtaining various phenotypes with different silencing efficiencies and exploring gene functions.

2 Materials and methods

2.1 Plant materials and growth conditions

Gossypium hirsutum L. cv. CCRI24 (Chinese Cotton Research Institute 24) seeds were germinated and grown in a greenhouse at the Institute of Cotton Research of Chinese Academy of Agricultural Sciences. The seedlings with the expanded cotyledons were prepared for agrobacterium infiltration. Infiltrated plants were grown in a culture room at 25 °C under white fluorescent light with a 16/8-h day/night cycle. All samples were harvested and immediately frozen in liquid nitrogen, and stored at −80 °C for future RNA extraction.

2.2 Recombinant VIGS vector construction and plant transformation

Candidate gene sequences were obtained from the cotton AADD genome database [21]. Two target sequences of *GhCLA1* were cloned into TRV vector to construct VIGS vectors pYL156-*GhCLA1-1* and pYL156-*GhCLA1-2*, respectively [22]. pYL156-*GhPDS-1*, pYL156-*GhPDS-2*, pYL156-*GhAOS1-1*, pYL156-*GhAOS1-2*, pYL156-*GhCXE1-1*, and pYL156-*GhCXE1-2* vectors were constructed in a similar manner. The cDNA sequences and primers used for quantitative real-time PCR (qRT-PCR) were listed in Tables S1 and S2, respectively. The above vectors were transformed into *Agrobacterium tumefaciens* strain GV3101 by freeze-thawing with liquid nitrogen. Spread of virus vectors was initiated by syringe-mediated agroinfiltration into two cotyledons of a cotton seedling [23]. Each experiment was performed in triplicate.

2.3 Selection of *S*fold filter parameters

$\Delta G_{disruption}$, DSSE, and AIS for target structure-based rational VIGS design were obtained through application of the *S*irna module of the *S*fold software (http://sfold.wadsworth.org).

To better analyze target sequences, filter criteria were selected as follows: (1) GC content between 30 % and 70 %; (2) target sequence avoided motifs AAAA, CCCC, GGGG, or UUUU, and long stretches of GCs (e.g., CCG CGGC, GCGCGGCG); (3) DSSE > 0 kcal/mol (asymmetry rule); (4) $\Delta G_{disruption} < 10$ kcal/mol; (5) AIS > –8.6 kcal/mol.

2.4 Guidelines used for analyzing target fragments by the *S*fold program

The *S*fold program was used to analyze the secondary structure of target fragment, the fragment length was limited to 250 base pairs (bp) for one time. Similar length of target fragment was selected for elimination of the target length influencing VIGS efficiency. If the fragment length was between 250 and 500 bp, they were divided into two parts, and examined twice.

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