MIGS as a Simple and Efficient Method for Gene Silencing in Rice

Xuelian Zheng, Lijia Yang, Qian Li, Linyi Ji, Aiting Tang, Lili Zhang, Kejun Deng, Jianping Zhou and Yong Zhang*

Department of Biotechnology, School of Life Sciences and Technology, Center for Informational Biology, University of Electronic Science and Technology of China, Chengdu, China

MiRNA-induced gene silencing (MIGS) technology is a special kind of RNA interference technology that uses miR173 to mediate the production of trans-acting siRNA (ta-siRNA) to achieve target gene silencing. This technique has successfully mediated the silencing of interested genes in plants such as Arabidopsis, tobacco, petunia, etc. In order to establish the MIGS technology system in monocots such as rice, we constructed the MIGS backbone vectors pZHY930, pZHY931, pZHY932, and pZHY933 with different promoters to regulate the expression of miR173 and miR173_ts. The rice OsPDS reporter gene was selected to compare the efficiency of four MIGS backbone vectors by the ratio of albino plants. The results showed that all the four backbone vectors could effectively mediate the target gene silencing, and pZHY932 showed highest efficiency up to 90%. Through MIGS silencing of endogenous OsROC5 and OsLZAY1 in rice, we successfully obtained rice mutant plants with rice leaf roll and tillering angles increasing, and further confirmed that MIGS backbone vector can efficiently mediate target gene silencing in rice. On the other hand, in order to verify the efficiency of MIGS-mediated multi-gene silencing in rice, we constructed two double-gene silencing vectors OsPDS and OsROC5, OsPDS and OsLZAY1, based on pZHY932 backbone vector. Double mutant rice plants with increased leaf and albino tiller angles. And we successfully obtained bladed leaf albino seedling and increased tillering angle albino seedling double-silencing mutations. We further constructed a MIGS-OsGBSS gene silencing vector and obtained rice materials with significantly reduced amylose content. This result indicated that MIGS could be an efficient method in monocots gene silencing and gene function analysis.

Keywords: rice, starch, MIGS, OsGBSS, amylose content

INTRODUCTION

RNA interference (RNAi) is a highly conserved biological process in which the 20–30 nt small RNA molecules (sRNA) inhibit gene expression by causing the degradation of specific mRNA molecules (Fire et al., 1998). Since its discovery in 1990s, RNAi has been widely used in the research and application of the functional genomics of animals and plants. Endogenous sRNA in living cells is widespread, according to the characteristics of its precursors; we divided sRNA into two main types: microRNA (miRNA) and small interfering RNA (siRNA). Both of them are length 20–25 nt, and produce by Dicer or DCL (Dicer-like) which are similar to RNase III (Agrawal et al., 2003). SiRNAs derive from fully complementary double stranded RNA and...
have a typical sequence diversity. The miRNAs processes by the pri-miRNA with the hairpin structure as well as typical sequence specificity (Chapman and Carrington, 2007; Xie and Qi, 2008; Voinnet, 2009). There are many kinds of siRNAs, according to the different types of the precursor sequence and the mechanism of formation, they can be divided into several types, include tasiRNA (trans-acting siRNA), natsiRNA (natural antisense transcript-derived siRNAs), hcsiRNA (heterochromatic siRNA), rasiRNA (repeat-associated siRNA) and others.


tasiRNA is a special siRNAs in higher plants, which generates from precursor transcripts by the specific miRNA/AGO (ARGONAUTE protein) complexes guided cleavage, and converts to dsRNA, and then processes into short 21 nt long RNA duplexes (Peragine et al., 2004; Vazquez et al., 2004; Axtell et al., 2006). tasiRNA is a combination of miRNA and siRNA silencing pathway, which has the characteristics of both siRNAs and miRNAs. Like siRNAs, tasiRNA undergo similar processing and arise from dsRNA, and their generation are both RDR6 (RNA-dependent RNA polymerase 6) dependent (Vázquez et al., 2004). However, tasiRNA like miRNAs binds with less sequence specificity to their targets, and does not require full sequence complementarity (Peragine et al., 2004; Yoshikawa et al., 2005). In Arabidopsis there are four families of tasiRNA genes (TAS loci), and different miRNAs corresponded to different TAS genes (TAS1, TAS2 corresponding miR173, TAS3 corresponding miR390, and TAS4 corresponding miR828) (Chapman and Carrington, 2007). TAS1 and TAS2 transcripts undergo miR173-guided cleavage by AGO1 at the 5’ end, dsRNA synthesis by RDR6, and generates the 21-nt siRNA by DCL4 to target complementary miRNAs. The TAS4 family is similar to that of TAS1 and TAS2, except for the guided miR828. Compared with those single mRNA binding family, TAS3 transcripts require the guide miR390 bind the transcript at two sites, and then cleave at the 3’ binding site only by AGO7 (Gasciolli et al., 2005; Axtell et al., 2006; Nakazawa et al., 2007; Martinez de Alba et al., 2013).

MIGS (miRNA-induced gene silencing) is an efficient technique to induce gene silencing in Arabidopsis, tobacco, soybean, and petunia (Felippes et al., 2012; Yao et al., 2015; Jacobs et al., 2016). By fusing a 22 nt long miR173 target site (miR173_ts) to the 5’ end of the target gene fragment, we constructed the MIGS vector through simply PCR reaction. Since the guide miR173 can incorporate into the same MIGS vector, the co-expression of guide miR173 and miR173_ts-target fragment lead MIGS can be used in any plant species. MIGS can also be used in multiple-gene silencing with a single vector by directly link the miR173_ts to different target fragments (Felippes et al., 2012). Compared with other gene silencing methods, VIGS requires full sequence complementarity (Peragine et al., 2004; Vazquez et al., 2004) and does not have a typical sequence diversity. The miRNAs processes by the pri-miRNA with the hairpin structure as well as typical sequence specificity (Chapman and Carrington, 2007; Xie and Qi, 2008; Voinnet, 2009). There are many kinds of siRNAs, according to the different types of the precursor sequence and the mechanism of formation, they can be divided into several types, include tasiRNA (trans-acting siRNA), natsiRNA (natural antisense transcript-derived siRNAs), hcsiRNA (heterochromatic siRNA), rasiRNA (repeat-associated siRNA) and others.

Here, we provide an efficient alternative to monocot gene silencing strategies. Based on tasiRNA MIGS principle and monocot specific expression cascade, we constructed three different monocot MIGS backbones. By using rice endogenous gene OsPDS (Rice phoyoene desaturase precursor, GenBank Accession No. AF049356) as visible reporter, we evaluated the efficiency of different backbones, and obtained OsPDS, OsROC5 (Rice outermost cell-specific 5 gene, GenBank Accession No. AB101648), OsLAZY1 (Rice shoot gravitropism gene, GenBank Accession No. DQ855268) silencing rice mutants through monocot MIGS system. Meanwhile, we detected the monocot multiple MIGS system by achieving the OsPDS-OsROC5 and OsPDS-OsLAZY1 dual mutant rice. Furthermore, we successfully knocked down the rice OsGBSS (Rice granule bound starch synthase 1 gene, GenBank Accession No. FJ235787) gene expression by MIGS technology, and obtained the transgenic rice less seeds with significant decrease in amylose content. These indicate that MIGS technology has a wide range of application prospects in rice research and breeding.

### MATERIALS AND METHODS

#### Plant Material and Growth Condition

**Oryza sativa** Japonica Group cultivar Nipponbare was used as the wild type and transformation host. Matured seeds were germinated for 2 days at 32°C in the dark. Germinated seeds were then transferred to soil, and seedlings were grown under 16 h light at 30°C 8 h dark at 22°C in greenhouse.

#### Plasmid Construction

Construction of MIGS backbone vector pZHY930 based on binary expression vector MIGS 2.1 (Felippes et al., 2012). The artificial mir173 sequence to insert the target gene 200–300 bp fragment. A similar method substitute for the ZmUbi, OsAct promoter to obtain pZHY931, pZHY932 and pZHY933 backbone vectors (Figure 1).

According to OsPDS gene sequence, the total RNA of rice was extracted from TRZol (Invitrogen, United States) method uses as template, ZLL008F (5’-GTGATTTTTCT CTACAAGCG AAGGTACCATT TTCTTCAGGAGAAGCATGG TTCTAAGATG-3’) and ZLL008R (5’-TTTATCCTATCTCCT ATATCTCGAGCCGGGATCAACTGTGTGTTGCAAAACAT AAGC-3’) uses as primers to amplify 289 bp OsPDS gene fragment by RT-PCR. The amplified OsPDS gene fragment introduced into the KpnI-SmaI double RE sites is downstream of the miR173_ts sequence to insert the target gene 200–300 bp fragment. A similar method substitute for the ZmUbi, OsAct promoter to obtain pZHY931, pZHY932 and pZHY933 backbone vectors (Figure 1).

According to OsPDS gene sequence, the total RNA of Nipponbare leaves extracts from TRZol (Invitrogen, United States) method uses as template, ZLL008F (5’-GTGATTTTTCT CTACAAGCG AAGGTACCATT TTCTTCAGGAGAAGCATGG TTCTAAGATG-3’) and ZLL008R (5’-TTTATCCTATCTCCT ATATCTCGAGCCGGGATCAACTGTGTGTTGCAAAACAT AAGC-3’) uses as primers to amplify 289 bp OsPDS gene fragment by RT-PCR. The amplified OsPDS gene fragment introduced into the KpnI-SmaI digested pZHY930 vector to obtain the MIGS expression vector pZHY930:OsPDS. The pZHY931::OsPDS, pZHY932::OsPDS, pZHY933::OsPDS vectors were constructed by similar method. The pZHY930::OsROC5, pZHY931::OsROC5, pZHY932::OsROC5, and pZHY933::OsROC5 vectors were constructed by primers ZLL026F (5’-GTGATTTTTCTCTACAAGCGAAGGTACCCTGCTGCGGTGTTCTAAGATG-3’) and ZLL026R (5’-TTTATCCTATCTCATCGAGCCGGGACTCAT CGTTGTCGGGCATTCTTTG-3’).
OsLAZY1, pZHY932::OsLAZY15, and pZHY933::OsLAZY1 vectors were constructed by primers ZLL027F (5′-GTGATTTTTCTCTACAGCGAAGGTA CACCTTCAATCCTCCACAG GAAG-3′) and ZLL027R (5′-TTTTCATCTCCATCTCATATC TCGAGGGAGGAGGCTCGGCTCACGTTGCTCAG-3′). The amplified OsGBSS gene fragment (ZLL014F: 5′-GTGA TTTTCTCTACAGCGAAAGTACATGCGGCTCACC ACGTCCAGCTGCGG-3′, ZLL014R: 5′-CCAATATTTTC ATCTTCAATCCTATCTGAGGCCGAGGACCAGCCAG GGGGAGGC-3′) introduced into the KpnI-SmaI digested pZHY932 vector to obtain the MIGS expression vector pZHY932::OsGBSS (Figure 5A).

In order to obtain the double gene MIGS vectors pZHY932::OsPDS::OsROC5 (Figure 4A) and pZHY932::OsPDS::OsLAZY1 (Figure 4B), the 5′ ends of the two gene fragments were fused with a mir173 ts sequence, respectively, then OsPDS::OsROC5 gene fragment (ZLL028F: 5′-TTTGCAACCCAGTTAGTGTATTTCCTCACAAGCGAGC CTGGCCGTGGTTCGCTTGTAGAGAAAAATCACATCAAC TGGTGTTGCAAA-3′) and OsPDS::OsLAZY1 gene fragment (ZLL029F: 5′-TTTGCACCTTTCACTTTATCTCACAAGCGAGCCTTG CCGCCTGGTTCGCTTGTAGAGAAAAATCACATCAACTG GGGGAGGC-3′) fused with OsActin (OsActin-F: 5′-CCTT GATTATGAGGAGGCTG-3′, OsActin-R: 5′-AAGTGATCTCCTCGTCATCAC-3′) fragment was used as an internal control to detect differences in expression levels of target gene in wild type and transgenic plants. Three biological replicates (three random-selected independent transgenic lines for each transgenic plants with different vectors) were examined to ensure reproducibility and each experiment was performed three times independently.

**Iodine Staining of Rice Endosperm and Microscope Observation**

The method used to rice starch granules iodine staining was performed according to Nakamura’s (Nakamura et al., 1995). Harvested rice seeds were husked, each strain was randomly selected for eight seeds to immerse in water for 24 h cut the soaked seeds horizontally with sharp blades. Absorb the prepared iodine solution to endosperm staining. After 2 min, remove the excess dye with a clean filter paper to ensure that the reaction time of the transgenic lines and the control is same. For microscopic observation, a little endosperm tissue of soaked seeds took with a tweezers to place on the slide. After 2 min, remove excess dye from the other end with a clean filter paper. For SEM observation, rice seeds were fractured across the short axis and dried completely under low pressure. The surface was sputter-coated with gold and observed by using a JMS-7500 scanning electron microscope at 5.0 KV (Li et al., 2005).

**Agrobacterium-Mediated Rice Transformation and Transgenic Positive Rice Detection**

Above expression vectors were transferred into *Agrobacterium tumefaciens* EHA105, respectively. Agrobacterium-mediated rice transformation was based on a previous method (Hiei et al., 1994; Tang et al., 2017). At the same time, *pCambia1301 transformation* was based on a previous method (Hiei et al., 1994; Zhu et al., 2008). For all the data analysis, three replicates (three random-selected independent transgenic lines for each transgenic plants with different vectors) were examined for statistical analysis. For microscopic observation, a little endosperm tissue of soaked seeds took with a tweezers to place on the slide. After 2 min, remove excess dye from the other end with a clean filter paper. For SEM observation, rice seeds were fractured across the short axis and dried completely under low pressure. The surface was sputter-coated with gold and observed by using a JMS-7500 scanning electron microscope at 5.0 KV (Li et al., 2005).

**Measurement of Starch Content and Amylose Content in the Endosperm**

Determination of total starch and soluble sugar by anthrone sulfuric acid method (Yemm and Willis, 1954; Li et al., 2011). Harvested rice grains were air-dry before analysis. Some dried kernels were selected, peel the seed coat, and everything that remained was dried together to constant weight at 60°C before the weight was determined. Then the dried endosperm was ground to powder for later study. Samples of the powdered endosperm prepared as mentioned above (50 mg) were extracted with 4 mL of 80% ethanol at 80°C for 40 min, followed by two extractions with 2 mL of 80% ethanol. The remaining pellets were dried at 60°C to remove the ethanol, and boiled for 10 min with 3 mL double-distilled water in 50 mL centrifuge tubes. Then the samples were cooled to room temperature, and 4 mL HClO₄ was added to decompose the starch. Starch in the paste hydrolyzed for 15 min, and the amount of soluble sugar was determined with the anthrone reagent using glucose as the standard. Starch content calculating used the formula: Starch content = G °0.9/DW °100%.

For amylose content detection, Milled samples (50 mg per sample) added 10 ml 1 mol/L KOH solution heated on the boiling water bath for 30 min. The amylose content was performed according to the procedure described in Hovenkamp-Hermelink et al. (1988), Zhu et al. (2008). For all the data analysis, three individual transgenic lines were performed and three individual
RESULTS

Monocot MIGS Vectors Based on Different Backbone

In order to make MIGS system work well in rice, we designed four different MIGS backbones and constructed according to MIGS vectors in Arabidopsis (Felippes et al., 2012). The MIGS pZHY930 backbone has a miR173_ts sequence followed by a multiple cloning site (MCS) for insertion of a fragment of target gene by sub-cloning, which between the CaMV 35S promoter and HSP terminator. An expression cassette with the miR173 precursor placed between the Arabidopsis UBQ10 constitutive promoters (AtUBQ10) and constructed OCT terminator into pZHY930, to ensure highly constitutive expression of miR173. The pZHY931 pZHY932 and pZHY933 backbones have different monocot constitutive promoters to maintain high expression rate of miR173 and miR173_ts. In pZHY931, the maize UBI constitutive promoter (ZmUBI) replaced the CaMV 35S promoter in pZHY930 to drive the expression of miR173_ts and target gene fragment. Comparing with pZHY931, the pZHY932 vector includes similar expression cassettes while the CaMV 35S promoter is in place of the Arabidopsis UBQ10 constitutive promoter. Furthermore, in pZHY933 we replaced CaMV 35S ZmUBI promoter to control expression of miR173_ts and target gene, while the rice actin promoter (OsACT) replaced AtUBQ10 to regulate the guide miR173 expressing. All these backbone vectors include an expression cassette with hygromycin resistance genes in plants (Figure 1).

Efficiency of OsPDS Gene Silencing by Using Different MIGS Vectors in Rice

For investigating the efficiency of different monocot backbones, we selected OsPDS gene as a target gene to construct the different MIGS vectors corresponding to pZHY930, pZHY931, pZHY932, and pZHY933 backbones, respectively. Four MIGS vectors with OsPDS target gene and pCambia1301 control vector were transformed into rice variety Nipponbare, respectively, regeneration transgenic rice plantlets were obtained from hygromycin resistant calli. Comparing normal green plantlets from pCambia1301 control vector, all the MIGS vectors regenerated PDS gene-silencing albino plantlets. It indicated that MIGS system functions effectively in rice (Figures 2b–e).

As expected, different MIGS backbones showed obviously different efficiency in OsPDS gene-silencing according to the proportion of white plantlets in all transgenic plantlets (albino rate), while pCambia1301 control plants were all green. As it was shown in Figure 2 and Table 1, albino rate of pZHY930::OsPDS backbone was about 10%, and the pZHY931::OsPDS and pZHY933::OsPD of is about 40%, and albino rate of pZHY932::OsPDS was dramatically increased to over 90%.

Further results of RT-PCR and semi quantitative analysis shows that the OsPDS mRNA level of pZHY930, pZHY931, pZHY932, and pZHY933 transgenic plants were significantly reduced, compared with that of pCambia1301 plants (Figure 2f), which consistent with the phenotype results. The ideal PDS gene-silencing phenotype and effectively inhibition of OsPDS transcriptional level exactly indicates that monocot MIGS vectors function well in rice.

ROC5 and LAZY1 Gene Silencing Using Monocot MIGS System in Rice

For further investigating the widely usage and high efficiency of monocot MIGS vectors, OsROC5 and OsLAZY1 genes controlling leaf rolling and tiller-spreading phenotype were selected to construct MIGS silencing vectors based on pZHY930, pZHY931, pZHY932, and pZHY933 backbones (Li et al., 2007; Takeshi and Moritoshi, 2007; Zou et al., 2011). All the MIGS vectors introduced into wild type Nipponbare rice via A. tumefaciens-mediated transformation, while the pCambia1301...
Zheng et al. An RNAi Method Based on tasiRNA in Rice

FIGURE 2 | Comparison of MIGS effect of different backbones on rice endogenesis PDS gene. MIGS induced down-regulation of OsPDS gene lead to albino phenotype. Comparing with pCambia1301 (a) control plants showed normal green, pZHY930::OsPDS (b), pZHY931::OsPDS (c), pZHY932::OsPDS (d), and pZHY933::OsPDS (e) MIGS plants showed different albino efficiency. The transgenic rice lines with pZHY932 backbone were almost albino and had the highest efficiency of gene silencing. (f) Semi-quantitative RT-PCR results showed that all the MIGS vectors could effectively reduce the expression level of endogenous OsPDS gene. Error bars indicate standard deviation (n = 3 independent experiments).

TABLE 1 | Comparison of miRNA-induced gene silencing (MIGS) effect on different backbones.

| Targeted locus | MIGS backbone | Number of PCR positive seedlings | Number of targeted locus with modified phenotype | MIGS efficiency |
|---------------|---------------|---------------------------------|-----------------------------------------------|-----------------|
| OsPDS         | pZHY930       | 64                              | 8                                             | 12.50%          |
|               | pZHY931       | 51                              | 15                                            | 29.41%          |
|               | pZHY932       | 63                              | 57                                            | 90.48%          |
|               | pZHY933       | 58                              | 23                                            | 39.66%          |
| OsROC5        | pZHY930       | 36                              | 3                                             | 8.30%           |
|               | pZHY931       | 36                              | 7                                             | 19.40%          |
|               | pZHY932       | 42                              | 37                                            | 88.10%          |
|               | pZHY933       | 39                              | 10                                            | 25.64%          |
| OsLAZY1       | pZHY930       | 38                              | 4                                             | 10.53%          |
|               | pZHY931       | 37                              | 9                                             | 24.32%          |
|               | pZHY932       | 38                              | 32                                            | 84.21%          |
|               | pZHY933       | 40                              | 11                                            | 27.50%          |

and the transcriptional level of MIGS-OsLAZY1 plantlets were significantly lower than the control plants (Figure 3D).

Different silencing efficacy of OsROC5 and OsLAZY1 genes in pZHY930, pZHY931, pZHY932, and pZHY933 vectors were similar to MIGS-OsPDS vectors, and was shown in Table 1. The pZHY930 backbone was the least efficient vector for MIGS gene silencing in monocot plants. And the pZHY932 backbone with CaMV 35S promoter controlling expression of the guide miR173 unit, combining ZmUbi promoter controlling expression of miR173.ts and target gene interference region, was the most efficient backbone in monocot plant, with efficiencies of around 90% (90.48% in OsPDS, 88.10% in OsROC5, 84.21% in OsLAZY1).

Multiple Gene Silencing Using Monocot MIGS in Rice

MiRNA-induced gene silencing could be used to simultaneously silence two different genes in Arabidopsis (Felippes et al., 2012).
We detected the multiple gene silencing effect on monocot MIGS system based on highest efficient backbone pZHY932. For dual gene silencing, two mir173_ts sequences followed by two different target gene fragments OsPDS-OsROC5 and OsPDS-OsLAZY1, respectively, were fused into a whole expression cassette, driven by ZmUBI promoter in pZHY932 (Figures 4A-B). Dual MIGS vectors were transformed into wild type Nipponbare and pZHY932 backbone vector used as control. Regeneration transgenic plantlets were confirmed by PCR detection. Expectedly, the transgenic plantlets of pZHY932::OsPDS::OsROC5 showed albino plant and outcurve rolling leaf at the same time, while the pZHY932 plants had green and flat leaves (Figure 4C). The pZHY932::OsPDS::OsLAZY1 plants displayed albino and spread-out phenotype as expected, and the pZHY932 control was normal (Figure 4D). RT-PCR results demonstrated that the OsPDS and OsROC5 genes expression level was significantly decrease simultaneously, and the similar results were obtained in OsPDS and OsLAZY1 dual gene silencing plants (Figures 4E,F). These results suggested that the monocot MIGS system successfully inhibit the double gene expression simultaneously in rice, and it could use not only in single gene silencing but also in multiple gene silencing in monocot plants.

**MIGS Silencing of OsGBSS Gene Affected Amylose Content in Rice Seeds**

As mentioned above, MIGS could mediate effective gene silencing of rice endogenous genes. We used MIGS technology to silence OsGBSS gene which regulated the synthesis of amylose content in rice to obtain low amylose rice seeds. The MIGS-OsGBSS vector pZHY932::OsGBSS was constructed based on the most efficient backbone pZHY932 and the first exon of the OsGBSS gene was used as the target fragment (Figure 5A). More than 50 transgenic rice lines were obtained by A. tumefaciens mediated transformation. Five transgenic positive lines performed by genomic PCR and the agronomic traits of these transgenic lines showed no difference from that of pZHY932 control. Three transgenic lines were selected for subsequent experiments. The expression of OsGBSS gene in the endosperm tissue of three lines were decreased significantly (Figure 5B). The results of iodine-stained showed that there was a significant difference in the color of endosperm between control and MIGS-OsGBSS seeds. The wild-type control showed typical blue-violet, while the seeds of MIGS-OsGBSS transgenic lines showed brown red or light brown (Figure 5D). The observation of iodine-stained starch granules by optical microscopy showed that the starch granules of control and MIGS-OsGBSS seeds. The wild-type control showed typical blue-violet, while the starch granules of MIGS-OsGBSS transgenic lines showed brown red or light brown, indicating that mainly containing amylopectin (Figure 5D). At the same time, the SEM was performed to observe whether the amylose content change had an effect on the structure of the starch granules by JMS-7500 SEM. The results showed that the starch granules were smooth and irregular in the MIGS gene silencing lines and wild type, there was no obvious difference (Data not shown).

Furthermore, the amylose content and amylopectin content of T1 generation seeds were quantitatively determined by dual wavelength method (Zhu et al., 2008). The results showed that the amylose content of the three gene silencing lines decreased significantly compared with the control seed. The amylose content of transgenic lines pZHY932::OsGBSS-08, pZHY932::OsGBSS-13, and pZHY932::OsGBSS-17 were 10.4, 12.0, and 9.9%, respectively, which were significantly lower than those of control 18.1% (Figure 5C). Thus, there was no significant difference in total starch content between the transgenic lines and controls (data not show). The above results indicated that
is sufficient to trigger the trans-linked to the gene-silenced target fragment, the guide miR173 silencing technique. As long as the 22nt miR173_ts target site MiRNA-induced gene silencing is a very efficient and simple gene generation. This technology provided a feasible solution for rice also down-regulated amylose content in the endosperm of T1 directional silencing of granulated starch synthase gene, but regulation of rice starch synthesis pathway, not only for the monocotyledonous plants had been successfully applied to the the amylose ratio in the seed. These results indicated that the MIGS system established in monocotyledonous plants had been successfully applied to the regulation of rice starch synthesis pathway, not only for the directional silencing of granulated starch synthase gene, but also down-regulated amylose content in the endosperm of T1 generation. This technology provided a feasible solution for rice breeding and improved starch quality.

**DISCUSSION**

MiRNA-induced gene silencing is a very efficient and simple gene silencing technique. As long as the 22nt miR173_ts target site linked to the gene-silenced target fragment, the guide miR173 is sufficient to trigger the trans-acting siRNA reaction, which in turn causes the silencing of the target gene. On the other hand, the size of the target gene fragment used in MIGS is about 200–500 nt, and the size of the fragment can effectively induce the silencing of the target gene, which makes the construction of MIGS vector very simple and requires only one step of PCR reaction achieve. In addition, MIGS can not only silence multiple homologous genes, but also very simply silence multiple different genes at the same time. It is only need to connect multiple MiR173_ts sequences to fragments of different target genes, respectively. Thus MIGS has been successfully used in tobacco, Arabidopsis, Medicago truncatula, soybean, petunia, and other plants for gene silenced (Felippes et al., 2012; Benstein et al., 2013; Imin et al., 2013; Yao et al., 2015; Jacobs et al., 2016). Felippe first studied miR173-mediated MIGS in Arabidopsis thaliana and tobacco, and MIGS can effectively induce single gene, double gene silencing in Arabidopsis and tobacco (Felippes et al., 2012). Benstein used MIGS to silence the phosphoglycerate dehydrogenase I (PGDH1) gene in A. thaliana, and the transgenic positive plants showed significant growth inhibition (Benstein et al., 2013). In addition, the C-terminal protein 1 (CEP1) gene of M. truncatula was successfully silenced by the MIGS2.1 vector (Imin et al., 2013). It has also been reported that the expression of CHS (chalcone synthase) and PDS (phytoene desaturase) gene was successfully inhibited by the MIGS technique in petunia, and the white albino plants were obtained (Vazquez et al., 2004; Yao et al., 2015).

We successfully established a simple and efficient MIGS system for single and multiple gene silencing in monocot plant rice. We constructed four different backbone vectors pZHY930, pZHY931, pZHY932, and pZHY933 by altering the promoters of the control guide miR173 and miR173_ts sequences, and the efficiency of induction of gene silencing in four backbone vectors had been compared and evaluated by using OsPDS, OsROCS, and OsLAZY1 genes as reporter genes. After comparing the data, we found that most of the PCR-positive plantlets of pZHY930 appeared normal green phenotype like control plants, miR173_ts transcription pattern in pZHY930 was not suitable for monocot expression in MIGS technique. In pZHY931 backbone, replacement of original CaMV 35S promoter of miR173_ts with monocot maize ZmUbi promoter, MIGS gene silencing rate increased significantly, which suggested that the monocot constitutive promoter was apparently more suitable for the miR173_ts and target gene expression in rice than the dicot promoter. In pZHY932 backbone, the controlling expression of guide miR173 was replaced by strong constitutive promoter CaMV 35S, which guaranteed the expression of miR173 was significantly increased and the gene-silencing effect was dramatically raised to 90%. It indicated that increasing the expression of guide miR173 by strong promoter 35S might be critical to improving gene silencing efficiency. However, it was unexpected that the pZHY933 backbone vector which had the monocot constitutive promoter OsACT to manipulate the guide miR173 expressing had not shown highly gene silencing efficiency as expected. The reason remained unclear and needed to further experimental analysis.

In addition to visible reporter gene OsPDS, we also successfully silenced the endogenous OsRoc5 and OsLAZY1

![FIGURE 5](image-url)
in the transgenic seed endosperm decreased from 24.53 to 16.38%, the down-regulation proportion reached 33.2%, which was significantly different from that in the control. It indicated that MIGS technology had effectively achieved the down-regulation of amyllose content in rice grains. Interestingly, although MIGS causes OsGBSS gene silencing and amyllose content decrease, there was no significant effect on total starch content. The decreased amyllose content might compensate by the increased amylpectin, which suggested that the synthesis network of rice starch was complex, it could be moderately regulated.

Compared with the efficiency of single gene silencing (60–95%), MIGS-induced OsGBSS gene down-regulation efficiency was lower than that of OsPDS, OsROC5, and OsLAZY1 genes. This should relate to the selection of MIGS interfering target sites, and the specificity of target gene fragments. There were also significant differences in the silencing efficiency of different species and different tasiRNA target sites. Generally, target sites with high sequence homology and specificity are more efficient for interfering certain gene. In addition, because of the large number of genes controlling starch traits, the complex metabolic regulation network, and the complementary function of gene family members, the effect of regulating a single gene of the starch synthesis pathway was often more complex than expected. This suggests that we could co-silence multiple genes in the starch synthesis pathway in further studies and regulate the starch composition more conveniently. MIGS could also combine with other co-expression techniques, such as gene stacking technology, to achieve precise regulation of gene expression in monocotyledonous plants.

**CONCLUSION**

RNAi as a highly efficient and specific technical method was widely used in animal and plant genomic function research. There were many types of RNAi technology; different RNA-mediated gene silencing efficiency was also different. MIGS was the interference technology by miRNA-mediated, tasiRNA binding degradation of the target gene that had emerged in plants in recent years. It had a simple construction steps and high efficiency in gene silence. It could simultaneously silence multiple unrelated genes or the same metabolic pathway of different regulatory genes to study the regulation of specific gene function in the organism. In the presence of effective tasiRNA, targeted gene silencing could be achieved in any species.

By changing different monocotyledon promoters to control expression of guide miR173 and miR173-ts sequences, we successfully constructed a backbone vector pZHY932 and MIGS system for efficient gene silencing in monocotyledonous plant rice. The single gene or double gene silencing of different rice endogenous genes verified the stability, efficiency and ease use of system in rice. At the same time, the rice-amyllose synthesis gene OsGBSS down regulated by the MIGS system, and its applicability in rice genetic improvement breeding was verified. This interesting, efficient and simple method could be widely used in monocotyledonous plants such as rice.
AUTHOR CONTRIBUTIONS

XZ and YZ conceived and designed the experiments. XZ and LZ generated all vectors. LZ, AT, LY, and QL performed the stable transgenic rice and analyzed the plants. XZ, AT, LJ, KD, and JZ performed the RT-PCR, amylose content analysis, and other experiments. YZ and XZ analyzed the data and wrote the paper. All authors read and approved the final manuscript.

REFERENCES

Agrawal, N., Dasaradhi, P. V., Mohomed, A., Malhotra, P., Bhatnagar, R. K., and Mukherjee, S. K. (2003). RNA interference: biology, mechanism and applications. Microbiol. Mol. Biol. Rev. 67, 657–688. doi: 10.1128/MMBR.67.4.657–685.2003

Axtell, M. J., Jan, C., Rajagopalan, R., and Bartel, D. P. (2006). A two-hit trigger for siRNA biogenesis in plants. Cell 127, 565–577. doi: 10.1016/j.cell.2006.09.032

Baba, T., Nishihara, M., Mizuno, K., Kawasaki, T., Shimada, H., Kobayashi, E., et al. (1993). Id entification, cDNA cloning, and gene expression of soluble starch synthase in rice (Oryza sativa L.) immature seeds. Plant Physiol. 103, 565–573.

Benstein, R. M., Ludewig, K., Wulfert, S., Wittek, S., Gigolashvili, T., Frrigemann, H., et al. (2013). Arabidopsis phosphoglucerase dehydrogenase1 of the phosphoserine pathway is essential for development and required for ammonium assimilation and tryptophan biosynthesis. Plant Cell 25, 5011–5029. doi: 10.1105/tpc.113.118992

Chapman, E. J., and Carrington, J. C. (2007). Specialization and evolution of endogenous small RNA pathways. Nat. Rev. Genet. 8, 884–896. doi: 10.1038/nrg2179

Craig, J., Lloyd, J. R., Tomlinson, K., Barber, L., Edwards, A., Wang, T. L., et al. (1998). Mutations in the gene encoding starch synthase 2 profoundly alter amylopectin structure in pea endosperm. Plant Cell 10, 413–426.

Edwards, A., Fulton, D. C., and Hylton, C. M. (1999). A combined reduction in activity of starch synthases II and III of potato has novel effects on the starch of tubers. Plant J. 17, 251–261. doi: 10.1046/j.1365-313X.1999.00371.x

Felippes, F. F., Wang, J. W., and Weigel, D. (2012). MIGS: miRNA induced gene silencing. Plant J. 70, 541–547. doi: 10.1111/j.1365-313X.2011.04896.x

Fire, A., Xu, S., Montgomery, M. K., Kostas, S. A., Driver, S. E., and Mello, C. C. (1998). Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. Nature 391, 806–811. doi: 10.1038/35888

Gascioll, V., Mallory, A. C., and Bartel, D. P. (2004). Endogenous trans-acting siRNAs regulate the accumulation of Arabidopsis Dicer-like protein DCL4 in vivo and functions in the trans-acting siRNA pathway. Plant Mol. Biol. 63, 777–785. doi: 10.1007/s11103-006-9125-8

Obana, Y., Omoto, D., and Kato, C. (2006). Enhanced turnover of transitory starch by expression of up-regulated ADP-glucose pyrophosphorylases in Arabidopsis thaliana. Plant Sci. 170, 1–11. doi: 10.1016/j.plantsci.2005.07.019

Peragine, A., Yoshihakawa, M., Wu, G., Albrecht, H. L., and Poethig, R. (2004). SGS3 and SGS2/SDE1/RDR6 are required for juvenile development and the production of trans-acting siRNAs in Arabidopsis. Genes Dev. 18, 2368–2379. doi: 10.1101/gad.1231804

Seferoglou, A. B., Koper, K., Can, F. B., Cevahir, G., and Kavakli, I. H. (2014). Enhanced heterotetrameric assembly of potato ADP-glucose pyrophosphorylase using reverse genetics. Plant Cell Physiol. 55, 1473–1483. doi: 10.1093/pcp/pcu078

Takeshi, Y., and Moriotoshi, I. (2007). Identification of the gravitropism-related Rice Gene LAYZ1 and elucidation of lazy1-dependent and -independent gravity signaling pathways. Plant Cell Physiol. 48, 678–688.

Tang, X., Lowder, L. G., Zhang, T., Malzahn, A. A., Zheng, X., Voytas, D. F., et al. (2017). A CRISPR-Cpf1 system for efficient genome editing and transcriptional repression in plants. Nat. Plants 3:17018. doi: 10.1038/s41477-017-018

Voinnet, O. (2009). Origin, biogenesis, and activity of plant microRNAs. Cell 136, 69–68. doi: 10.1016/j.cell.2009.01.046

Xie, Z., and Qi, X. (2008). Diverse small RNA-directed silencing pathways in plants. Biochim. Biophys. Acta 1779, 720–724. doi: 10.1016/j.bbadis.2008.02.009

Yao, H., Zhang, B., Qin, X., Li, M., and Guo, Y. (2015). Investigation of a miRNA-Induced gene silencing technique in petunia reveals alterations in mir173 precursor processing and the accumulation of secondary sirnas from endogenous genes. PLoS One 10:e0144909. doi: 10.1371/journal.pone.0144909

Yemm, E. W., and Willis, A. J. (1954). The estimation of carbohydrates in plant extracts by the anthrone. Biochem. J. 57, 508–514. doi: 10.1014/bj/050 70508

Li, N., Zhang, S., Zhao, Y., Li, B., and Zhang, J. (2011). Over-expression of AGPase genes enhances seed weight and starch content in transgenic maize. Planta 233, 241–250. doi: 10.1007/s00425-010-1296-5

Li, P., Wang, Y., Qian, Q., Fu, Z., Wang, M., Zeng, D., et al. (2007). LAZY1 controls rice shoot gravitropism through regulating polar auxin transport. Cell Res. 17, 402–410. doi: 10.1038/cr.2007.38

FUNDING

This work is supported by grants including the National Natural Science Foundation of China (31371682 and 31771486), the Sichuan Youth Science and Technology Foundation (2017Q0005), and the Fundamental Research Funds for the Central Universities (ZYGX2016J119 and ZYGX2016J122) to YZ and XZ.
Yoshikawa, M., Peragine, A., Park, M. Y., and Poethig, R. S. (2005). A pathway for the biogenesis of trans-acting siRNAs in Arabidopsis. *Genes Dev.* 19, 2164–2175. doi: 10.1101/gad.1352605

Zhu, T., Jackson, D. S., and Wehling, R. L. (2008). Comparison of amylase determination methods and the development of a dual wavelength iodine binding technique. *Cereal Chem.* 85, 51–58. doi: 10.1094/CCHEM-85-1-0051

Zou, L. P., Sun, X. H., Zhang, Z. G., Liu, P., Wu, J. X., Tian, C. J., et al. (2011). Leaf rolling controlled by the homeodomain leucine zipper class IV Gene Roc5 in Rice. *Plant Physiol.* 156, 1589–1602. doi: 10.1104/pp.111.176016

**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2018 Zheng, Yang, Li, Ji, Tang, Zang, Deng, Zhou and Zhang. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.