Received: 24 Dec 2023 | Revised: 18 Jan 2024 | Accepted: 01 Feb 2024

Electronic Journal of Plant Breeding

Research Article

Disruption of *miR156* binding site of the *GW8* gene affects rice grain morphology in the variety ASD16

A. Shanthinie[1], L. Arul[1], S. Varanavasiappan[1], K. K. Kumar[1], S. Manonmani[2], P. Jeyakumar[3], E. Kokiladevi[1]* and D. Sudhakar[1]

[1]Department of Plant Biotechnology, Centre for Plant Molecular Biology and Biotechnology, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India.
[2]Department of Rice, Centre for Plant Breeding and Genetics, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India.
[3]Department of Plant Physiology, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India.
***E-Mail:**kokiladevi@tnau.ac.in

Abstract
Rice grain width is intricately governed by multiple aspects of grain development, such as cell division and expansion, thereby affecting overall grain morphology. Several key genes were identified and studied for their role in regulating grain width. *OsSPL16*/*GW8* is a positive regulator of grain width by boosting grain weight, a major determinant of grain yield. The *GW8* mRNA transcripts are cleaved by *miR156*, leading to reduced gene expression. In this study, we employed the CRISPR/Cas9 system to generate *gw8* mutants with a disrupted *GW8-miR156* binding site to enhance both grain morphology and yield in the ASD16 rice genetic background. *Agrobacterium*-mediated rice genetic transformation resulted in the generation of 25 putative *gw8* mutants. Sanger sequencing revealed the presence of mutations within and outside the *GW8-miR156* module. Notably, plants harboring mutations disrupting the *GW8-miR156* module exhibited an altered grain morphology with awn and poor grain set. Further research is needed to unravel the pleiotropic effects associated with the *GW8-miR156* module in rice.

Keywords: Rice, Grain width, *SPL16* gene, *GW8*, *miR156*, CRISPR/Cas9.

INTRODUCTION

Rice is an important cereal crop widely consumed by the majority of the global population. As the world's population continues to expand, there is a pressing need to increase grain yields to meet rising food demands. Rice grain yield depends on three primary components, panicles per plant, grains per panicle, and grain weight. The rice grain weight is mainly determined by length, width, and thickness (Chen *et al*., 2021; Wang *et al*., 2022). The structure of the rice grain is complex, consisting of the embryo and endosperm surrounded by a thin seed coat and enveloped by a hull. The endosperm, the main edible part of the rice plant, constitutes the majority of the mature seed, storing starches and essential nutrients. Moreover, the spikelet hull, comprising the palea and lemma, not only acts as a protective covering but also influences the grain-filling capacity (Juliano and Tuaño, 2019). Recent research has provided insights into the underlying mechanisms governing the grain size of rice. The first QTL to be successfully cloned in rice was *GRAIN WIDTH 2* (*GW2*), which encodes a RING-type E3 ubiquitin ligase that negatively regulates cell count and size. A deletion of 67 bases in the 3' splice site of the 6th intron of the *GW2* gene resulted in the development of wider grains and sturdier culms in rice, which in turn led to enhanced grain yield (Yamaguchi *et al*., 2020). Additionally, the downregulation of *OsGW2* in *indica* rice genotypes through RNAi technology resulted in wider and heavier grains (Verma *et al*., 2021). The *GS3* gene, located in

the pericentromeric region of the third chromosome, also governs the size of rice grains. Loss-of-function mutations in the N-terminal region of the *GS3* protein result in the production of slender rice grains (Takano-Kai *et al*., 2009). The *WIDE AND THICK GRAIN 1* (*WTG1*) gene plays a crucial role in regulating the size and shape of grains through cellular expansion mechanisms. When *WTG1* is over-expressed, it leads to the development of narrow, thin, and elongated grains. In contrast, the γ-induced *wtg1* mutation results in wider and shorter grains with an increased abundance of grains per panicle (Huang *et al*., 2017). The *GSE5* gene is recognized as a prominent QTL influencing rice chalkiness, demonstrating pleiotropic effects on both chalkiness and grain shape. Through genetic analysis, it was determined that *GSE5* operates as a dominant gene affecting grain length and as a semi-dominant gene influencing grain width and chalkiness. (Jiang *et al*., 2022). The *GSE9* gene contributes to the grain morphological differences between *indica* and *japonica* rice varieties (Chen *et al*., 2023).

The *SQUAMOSA-PROMOTER BINDING PROTEIN-LIKE (SPL)* genes are a specialized group of transcription factors that play multiple roles in various developmental processes, including those related to phase transitions in reproductive structures such as flowers and grains (Xie *et al*., 2006; Li *et al*., 2022; Liu *et al*., 2016). Specifically, two *SPL* genes, *OsSPL13* and *OsSPL16,* have been found to regulate grain size and shape in rice *(Xie et al., 2006)*. *OsSPL13 or GLW7* is involved in regulating the length and thickness of rice grains but does not influence grain width (Si *et al*., 2016). The *OsSPL16* or *qGW8* (*Grain Width 8)* gene expedites the grain filling and increases grain width. *OsSPL16* enhances latitudinal expansion through the stimulation of cell proliferation but inhibits longitudinal expansion by suppressing cell elongation (Wang *et al*., 2012). *OsSPL16* is also one of the 11 *SPL* genes that are potential targets of a highly conserved microRNA, *OsmiR156* (Wang *et al*., 2012; Wang and Zhang, 2017). Elevated levels of mature *miR156* transcripts lead to a substantial decrease in *OsSPL16* expression, ultimately causing a reduction in the size of rice grains. However, transgenic overexpression of *SPL16/GW8* is shown to enhance both cell division and grain filling, leading to positive effects on grain width and overall rice yield (Wang *et al*., 2012). Additionally, *SPL16*/*GW8* regulates grain size by binding to the promotor of the *GW7* gene and repressing *GW7* gene expression (Wang *et al*., 2015).

The mechanism of CRISPR/Cas9 involves directing the Cas9 enzyme to the target region in the genome through a small-guide RNA (sgRNA), upon reaching its designated target within the genome, the Cas9 enzyme initiates a break in both strands of the DNA. Subsequently, this double-stranded break is repaired by the repair machinery inherent to the cell, resulting in the installation of specific modifications to the DNA (Jiang and Doudna, 2017). The CRISPR/Cas9 system possesses the ability to expedite the breeding process and introduce desirable traits into crops by optimizing the shape, size, and quality of crops, and it is a promising tool for crop improvement (Jaganathan *et al*., 2018). In this study, we attempted to generate *gw8* mutants with mutations in the *miR156* binding site to enhance both grain morphology and yield in the rice variety ASD16.

MATERIALS AND METHODS

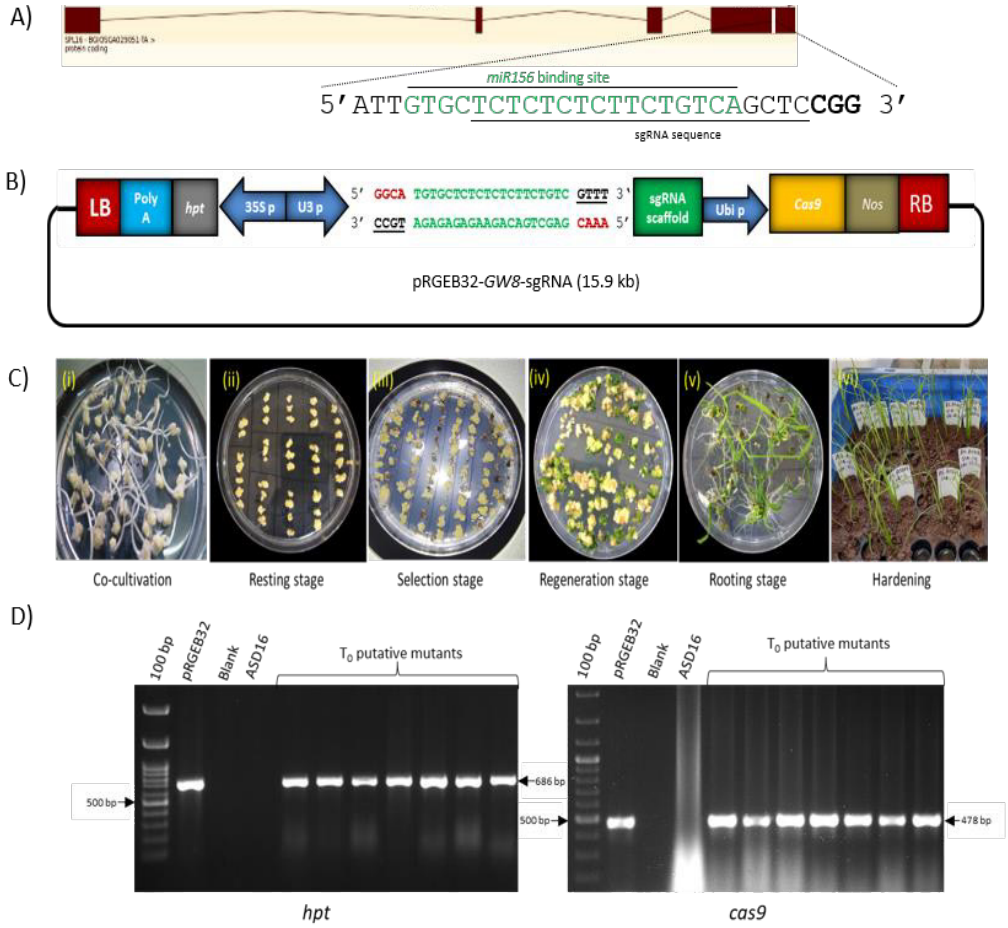
Development of the CRISPR/Cas9 construct: The gene sequence and structure information of *OsSPL16/GW8* (BGIOSGA029051) were retrieved from the Ensembl Plants database. The *GW8* gene is composed of four exons with a CDS of 1,248 bp and a translation length of 415 amino acids. The sgRNA targeting the *miR156* binding site present in the fourth exon was designed (**Fig. 1A**) using the web tool CRISPR-GE (Xie *et al*., 2017). Suitable adaptor sequences were added at the 5' end of the oligos (**Fig. 1A**). The annealed oligos were ligated into the linearized binary vector pRGEB32 (Addgene plasmid # 63142; http://n2t.net/addgene:63142; RRID: Addgene_63142) at the *Bsa*I site. The recombinant plasmid harboring the *GW8*-sgRNA expression cassette (**Fig. 1B**) was transformed into *E. coli*, and subsequently mobilized into the *Agrobacterium* and used as the inoculum for the co-cultivation.

Genetic transformation:The putative mutants were generated by using immature embryos of ASD16 as explants, employing the *Agrobacterium*-mediated genetic transformation method suggested by Hiei and Komari (2008) with minor modifications (Nithya *et al*., 2020; Arulganesh *et al*., 2021; Kumam *et al.,* 2021) (**Fig. 1C**).

Genotyping of putative *gw8* mutants: Genomic DNA was isolated from wild-type (WT) and putative gw8 mutants. The PCR confirmation of the presence of exogenous genetic elements such as the *hpt* and *cas9* genes in the T_0 plants was performed using gene-specific primers (**Table 1**). The target protospacer region was amplified using target-specific primers (**Table 1)** and Sanger sequenced (Biokart, Bengaluru). The CRISPR edits were analyzed using the online tool DSDecodeM (Liu *et al*., 2015). The T_0 plants with mutations progressed through T_1 and T_2 generations to confirm the stable inheritance of the mutation.

RESULTS AND DISCUSSION

Previous studies have indicated that modifying the *GW8-miR156* regulatory module has the potential for developing rice varieties with enhanced grain yield and improved grain quality (Wang *et al*., 2012). However, there are no reports on targeted editing of the *miR156* binding site present in the *OsSPL16/GW8* gene. Hence, we attempted to disrupt the *GW8-miR156* binding site through the CRISPR/Cas9 system to study changes in grain morphology.



E)

ASD16-WT	GTGCTCTCTCTCTTCTGTCAGCTCCGGCGA	-	-
gw8-1/1-3-4	GTGCTCTCTCTCTTCTGTCAG**A**CTCCGGCGA	1i ; Homozygous	Mutation outside *miR156* binding site
gw8-10/2-5-2	GTGCTCTCTCTCTTCTGTCAG**T**CTCCGGCGA	1i ; Homozygous	
gw8-12/1-1-3	GTGCTCTCTCTCTTCTGTCAG**G**CTCCGGCGA	1i; Homozygous	
gw8-5/1-3-1	GTGCTCTCTCTCTTCTGTCA-CTCCGGCGA	1d; Homozygous	
gw8-4/1-4-2	GTGCTCTC----------CGGCGA	16d; Homozygous	Mutation disrupting *miR156* binding site
gw8-24/2-2-1	GTGCTCTCTCTCT**C**CTG**C**C----------A	2s,10d; Homozygous	

Fig. 1. CRISPR/Cas9-mediated editing of *GW8-miR156* module

A) Gene structure and location of sgRNA target site in *SPL16/GW8* gene. B) Physical map of pRGEB32-*GW8*-sgRNA construct. C) *Agrobacterium*-mediated rice genetic transformation of ASD16. D) PCR analysis for the presence of *hpt* and *Cas9* genes in representative edited events. E) DNA traces of target region in homozygous *gw8* mutants (T_2). Nucleotides highlighted in green represent the *miR156* binding site, 'i' denotes insertion, 'd' denotes deletion, and 's' denotes substitution.

Molecular characterization of edited plants: A short-guide RNA targeting the *miR156* binding site present in the fourth exon of the *GW8* gene was cloned and mobilized into *Agrobacterium*. A total of 25 independent transgenic events were generated in cv. ASD16 with a transformation efficiency of 2.53% (**Table 2**). All the putative T_0 events were positive for the *hpt* and *cas9* genes (**Table 2, Fig. 1D**). Sanger sequencing of the target region revealed the presence of mutations in twelve mutants, indicating a mutation efficiency of 48% (**Table 2**). All twelve events were advanced to the T_1 generation, and six events were identified to be homozygous mutants. Subsequently,

Table 1. Primers used in this study

Name of the gene/ selection marker	Forward and reverse primers (5'--- 3')	PCR temperature profile		Amplicon size
hpt	hpt F: GCTGTTATGCGGCCATTGGTC hpt R: GCCTCAGAAGAAGATGTTG	94 °C for 5 mins 94 °C for 45 secs 58 °C for 45 secs 72 °C for 45 secs 72 °C for 10 mins	30 cycles	686 bp
cas9	cas9 F: CTTCTGGCGGTTCTCTTTAG cas9 R: TGCTGTTTGATCCGTTGTTC	95 °C for 5 mins 95 °C for 45 secs 52 °C for 45 secs 72 °C for 45 secs 72 °C for 10 mins	30 cycles	478 bp
GW8-Exon 4	GW8 F: AACCGAGGAGAGCCCATACT GW8 R: CATGAGAACGGCAGAGACGA	95 ˚C for 5 mins 95 ˚C for 45 secs 59 ˚C for 45 secs 72 ˚C for 45 secs 72 ˚C for 10 mins	30 cycles	678 bp

Table 2. *Agrobacterium*-mediated transformation of ASD16

Variety	Number of co-cultivated embryos	Number of events regenerated	Transformation efficiency# (%)	Number of *hpt & cas9* positive events	Number of plants with mutation	Mutation efficiency* (%)
ASD16	986	25	2.53	25	12	48

#Transformation efficiency = (Number of events regenerated/ Total number of embryos co-cultivated) × 100
*Mutation efficiency = (Number of events with mutation/ Total number of events regenerated) × 100

these six events, comprising four with indels outside the *miR156* binding site and two with indels within the *miR156* binding site, were further advanced to the T_2 generation. Sanger sequencing of the target region of T_2 plants revealed that the mutations were inherited, as observed in the T_1 generation. The nucleotide changes outside the *miR156* binding site were limited to one base pair in all four mutants. Conversely, the two mutants with mutations within the *miR156* binding site had larger deletions of 16 bp and 10 bp (**Fig. 1E, Table 3**).

Phenotypic alterations in grain morphology: Plants with mutations outside the *miR156* binding site displayed grain morphology similar to the wild-type. Conversely, plants with mutations disrupting the *miR156* binding site displayed a distinct grain morphology characterized by awn. The grain length in mutants increased to 9.7 mm from 7.1 mm (ASD16-WT), while the average grain width in mutants decreased to 2.6 mm from 3.2 mm (ASD16-WT) (**Fig. 2**). This resembles the traits found in wild rice, where it yields fewer lengthy grains accompanied by extended awns that play a vital role in both seed dispersal and protecting seeds from being consumed by granivores (Hua *et al*., 2015). Through millennia of selective breeding, plant breeders have selected and cultivated rice plants with shorter and awnless grains, more grains per panicle, all of which contribute to the ease of post-harvest processing and storage (Bessho-Uehara *et al*., 2021).

Furthermore, a reduced number of filled grains per panicle (approximately 20 grains per panicle) and an increased presence of chaffy spikelets were observed in the plants with mutations within the *miR156* binding site. The plants with mutations outside the *miR156* binding site also exhibited reduced panicle length and had poor grain set (**Fig. 2**). Awns play a role in altering the structure of spikelets, leading to increased spikelet sterility and grain enlargement. This phenomenon can be attributed to the distribution of photosynthetic assimilates toward the rapidly growing longer awns in rice, resulting in a reduction in spikelet fertility. Consequently, awns compete for assimilates during the growth of the ovary, resulting in a further decrease in rice grain yield (Rebetzke *et al*., 2016). This is in contrast with other cultivated cereals that retain longer awns, such as wheat and barley. In these grains, awns serve as important photosynthetically active organs that aid in grain filling, accounting for 30–50% of the total grain weight (Lu and Lu, 2004).

Awn development is a complex trait regulated by many genes, including *An1*, *An2, Laba1,* and many others (Luong *et al*., 2022; Luo *et al*., 2013; Hua *et al*., 2015). *GAD1* encoding EPFL peptide regulates grain number, grain length, and awn development in rice. The non-functional *gad1* gene resulted in an increased number of grains per panicle, shorter grains, and awnless phenotypes in cultivated rice (Jin *et al*., 2023). There is no direct evidence linking the *GW8-miR156* module to awn

https://doi.org/10.37992/2024.1501.001

Table 3. Inheritance of mutations in the T_1 progenies of *gw8* mutants

T_0 generation				T_1 generation			
S. No.	**Event I.D**	**Nucleotide traces**	**Mutation***	**Event I.D#**	**Nucleotide traces**	**Mutation***	
	ASD16-WT	WT: GTGCTCTCTCTCTTCTGTCAGCTCCGGCGA	-	ASD16-WT	WT: GTGCTCTCTCTCTTCTGTCAGCTCCGGCGA	-	
1	gw8-1/1	A1: GTGCTCTCTCTCTTCTGTCAGACTCCGGCGA A2: GTGCTCTCTCTCTTCTGTCAGACTCCGGCGA	1i; Homozygous	**gw8-1/1-3**	A1: GTGCTCTCTCTCTTCTGTCAGACTCCGGCGA A2: GTGCTCTCTCTCTTCTGTCAGACTCCGGCGA	1i; Homozygous	
2	gw8-2/1	A1: GTGCTCTCTCTCTTCTGTCAGCTCCGGCGA A2: GTGCTCTCTCTCTTCTGTCAGCTCCGGCAA	WT/1s; Monoallelic	gw8-2/1-1	A1: GTGCTCTCTCTCTTCTGTCAGCTCCGGCGA A2: GTGCTCTCTCTCTTCTGTCAGCTCCGGCAA	WT/1s; Monoallelic	
				gw8-2/1-4	A1: GTGCTCTCTCTCTTCTGTCAGCTCCGGCGA A2: GTGCTCTCTCTCTTCTGTCAGCTCCGGCAA	WT/1s; Monoallelic	
3	gw8-5/1	A1: GTGCTCTCTCTCTTCTGTCA--CTCCGGCGA A2: GTGCTCTCTCTCTTCTGTCAGACTCCGGCGA	1d/1i; Biallelic	gw8-5/1-2	A1: GTGCTCTCTCTCTTCTGTCA--CTCCGGCGA A2: GTGCTCTCTCTCTTCTGTCAGACTCCGGCGA	1d/1i; Biallelic	
				gw8-5/1-3	A1: GTGCTCTCTCTCTTCTGTCA-CTCCGGCGA A2: GTGCTCTCTCTCTTCTGTCA-CTCCGGCGA	1d; Homozygous	
				gw8-5/1-4	A1: GTGCTCTCTCTCTTCTGTCA--CTCCGGCGA A2: GTGCTCTCTCTCTTCTGTCAGACTCCGGCGA	1d/1i; Biallelic	
4	gw8-6/2	A1: GTGCTCTCTCTCTTCTGTCAG-CTCCGGCGA A2: GTGCTCTCTCTCTTCTGTCAGACTCCGGCGA	WT/1i; Monoallelic	gw8-6/2-1	A1: GTGCTCTCTCTCTTCTGTCAG-CTCCGGCGA A2: GTGCTCTCTCTCTTCTGTCAGACTCCGGCGA	WT/1i; Monoallelic	
5	gw8-10/2	A1: GTGCTCTCTCTCTTCTGTCAG-CTCCGGCGA A2: GTGCTCTCTCTCTTCTGTCAGTCTCCGGCGA	WT/1i; Monoallelic	gw8-10/2-3	A1: GTGCTCTCTCTCTTCTGTCAG-CTCCGGCGA A2: GTGCTCTCTCTCTTCTGTCAGTCTCCGGCGA	WT/1i; Monoallelic	Mutation outside *miR156* binding site
				gw8-10/2-5	A1: GTGCTCTCTCTCTTCTGTCAGTCTCCGGCGA A2: GTGCTCTCTCTCTTCTGTCAGTCTCCGGCGA	1i; Homozygous	
6	gw8-12/1	A1: GTGCTCTCTCTCTTCTGTCAGGCTCCGGCGA A2: GTGCTCTCTCTCTTCTGTCAGACTCCGGCGA	1i/1i; Biallelic	**gw8-12/1-1**	A1: GTGCTCTCTCTCTTCTGTCAGGCTCCGGCGA A2: GTGCTCTCTCTCTTCTGTCAGGCTCCGGCGA	1i; Homozygous	
7	gw8-15/1	A1: GTGCTCTCTCTCTTCTGTCAG-CTCCGGCGA A2: GTGCTCTCTCTCTTCTGTCAGTCTCCGGCGA	WT/1i; Monoallelic	gw8-15/1-1	A1: GTGCTCTCTCTCTTCTGTCAG-CTCCGGCGA A2: GTGCTCTCTCTCTTCTGTCAGTCTCCGGCGA	WT/1i; Monoallelic	
8	gw8-18/1	A1: GTGCTCTCTCTCTTCTGTCAG-CTCCGGCGA A2: GTGCTCTCTCTCTTCTGTCAGTCTCCGGCGA	WT/1i; Monoallelic	gw8-18/1-3	A1: GTGCTCTCTCTCTTCTGTCAG-CTCCGGCGA A2: GTGCTCTCTCTCTTCTGTCAGTCTCCGGCGA	WT/1i; Monoallelic	
9	gw8-19/1	A1: GTGCTCTCTCTCTTCTGTCAGACTCCGGCGA A2: GTGCTCTCTCTCTTCTGTCAGTCTCCGGCGA	1i/1i; Biallelic	gw8-19/1-1	A1: GTGCTCTCTCTCTTCTGTCAGTCTCCGGCGA A2: GTGCTCTCTCTCTTCTGTCAGTCTCCGGCGA	1i; Homozygous	
				gw8-19/1-4	A1: GTGCTCTCTCTCTTCTGTCAGACTCCGGCGA A2: GTGCTCTCTCTCTTCTGTCAGTCTCCGGCGA	1i/1i; Biallelic	
10	gw8-20/2	A1: GTGCTCTCTCTCTTCTGTCAGACTCCGGCGA A2: GTGCTCTCTCTCTTCTGTCAGACTCCGGCGA	1i; Homozygous	gw8-20/2-1	A1: GTGCTCTCTCTCTTCTGTCAGACTCCGGCGA A2: GTGCTCTCTCTCTTCTGTCAGACTCCGGCGA	1i; Homozygous	
11	gw8-4/1	A1: GTGCTCTC----------CGGCGA A2: GTGCTCTCTCTCTTCTGTCA-CTCCGGCGA	16d/1d; Biallelic	gw8-4/1-1	A1: GTGCTCTCTCTCTTCTGTCA-CTCCGGCGA A2: GTGCTCTCTCTCTTCTGTCA-CTCCGGCGA	1d; Homozygous	
				gw8-4/1-2	A1: GTGCTCTC----------CGGCGA A2: GTGCTCTCTCTCTTCTGTCA-CTCCGGCGA	16d/1d; Biallelic	Mutation disrupting *miR156* binding site
				gw8-4/1-4	A1: GTGCTCTC----------CGGCGA A2: GTGCTCTC----------CGGCGA	16d; Homozygous	
12	gw8-24/2	A1: GTGCTCTCTCTCTTCTGTCAGCTCCGGCGA A2: GTGCTCTCTCTCTCCTGCC----------A	WT/2s,10d; Monoallelic	**gw8-24/2-2**	A1: GTGCTCTCTCTCTCCTGCC----------A A2: GTGCTCTCTCTCTCCTGCC----------A	2s,10d; Homozygous	

*Nucleotides highlighted in green represents the *miR156* binding site, 'i' denotes insertion, 'd' denotes deletion, and 's' denotes substitution.
#Mutants highlighted in bold were forwarded to T_2 generation.

Fig. 2. Phenotypic observation of *gw8* mutants

A) Panicle morphology. B) Grain morphology

formation in rice. However, further research is needed to elucidate the signaling networks through which *SPL16/GW8* influences awn development in rice.

Hence, we conclude that the targeted editing of the *GW8-miR156* module *via* the CRISPR/Cas9 tool resulted in the development of *gw8* mutants exhibiting wild rice characteristics. Over-expression of *SPL* genes is often associated with negative pleiotropic effects. Hence, novel strategies have to be adopted for fine-tuning the expression levels of *SPL* genes for positive regulation.

ACKNOWLEDGEMENT

The authors thank the Department of Plant Biotechnology, Centre for Plant Molecular Biology and Biotechnology, Tamil Nadu Agricultural University, Coimbatore for providing facilities.

REFERENCES

Arulganesh, T., Kumam, Y., Kumar, K. K., Arul, L., Kokiladevi, E., Nakeeran, S., Varanavasiappan, S., Manonmani, S. and Sudhakar, D. 2021. Genome editing of elite rice cultivar CO51 for bacterial leaf blight resistance. *Electronic Journal of Plant Breeding*, **12**: 1060-1068. [Cross Ref]

Bessho-Uehara, K., Yamagata, Y., Takashi, T., Makino, T., Yasui, H., Yoshimura, A. and Ashikari, M. 2021. Exploring the loci responsible for awn development in rice through comparative analysis of all AA genome species. *Plants*, **10**(4): 725. [Cross Ref]

Chen, K., Łyskowski, A., Jaremko, Ł. and Jaremko, M. 2021. Genetic and molecular factors determining grain weight in rice. *Frontiers in Plant Science*, **12:** 605799. [Cross Ref]

Chen, R., Xiao, N., Lu, Y., Tao, T., Huang, Q., Wang, S., Wang, Z., Chuan, M., Bu, Q., Lu, Z., Wang, H., Su, Y., Ji, Y., Ding, J., Gharib, A., Liu, H., Zhou, Y., Tang, S., Liang, G., Zhang, H., Yi, C., Zheng, X., Cheng, Z., Xu, Y. and Yang, Z. 2023. A de novo evolved gene contributes to rice grain shape difference between *indica* and *japonica*. *Nature Communications*, **14**(1): 5906. [Cross Ref]

Hiei, Y. and Komari, T. 2008. *Agrobacterium*-mediated transformation of rice using immature embryos or calli induced from mature seed. *Nature protocols*, **3**(5): 824-834. [Cross Ref]

Hua, L., Wang, D. R., Tan, L., Fu, Y., Liu, F., Xiao, L., Zhu, Z., Fu, Q., Sun, X., Gu, P., Cai, H., McCouch, S. R.

and Sun, C. 2015. *LABA1*, a domestication gene associated with long, barbed awns in wild rice. *The Plant Cell*, **27**(7): 1875-1888. [Cross Ref]

Huang, K., Wang, D., Duan, P., Zhang, B., Xu, R., Li, N. and Li, Y. 2017. WIDE AND THICK GRAIN 1, which encodes an otubain-like protease with deubiquitination activity, influences grain size and shape in rice. *The Plant Journal*, **91**(5): 849-860. [Cross Ref]

Jaganathan, D., Ramasamy, K., Sellamuthu, G., Jayabalan, S. and Venkataraman, G. 2018. CRISPR for crop improvement: an update review. *Frontiers in plant science*, **9**: 985. [Cross Ref]

Jiang, F. and Doudna, J. A. 2017. CRISPR–Cas9 structures and mechanisms. *Annual review of biophysics*, **46**: 505-529. [Cross Ref]

Jiang, L., Zhong, H., Jiang, X., Zhang, J., Huang, R., Liao, F., Deng, Y., Liu, Q., Huang, Y., Wang, H., Tao, Y. and Zheng, J. 2022. Identification and pleiotropic effect analysis of *GSE5* on rice chalkiness and grain shape. *Frontiers in Plant Science*, **12:** 814928. [Cross Ref]

Jin, J., Xiong, L., Gray, J. E., Hu, B. and Chu, C. 2023. Two awn-development-related peptides, *GAD1* and *OsEPFL2*, promote seed dispersal and germination in rice. *Molecular Plant*, **16**(3): 485-488. [Cross Ref]

Juliano, B. O. and Tuaño, A. P. P. 2019. Gross structure and composition of the rice grain. *rice* (pp. 31-53). Elsevier. [Cross Ref]

Kumam, Y., Rajadurai, G., Kumar, K. K., Varanavasiappan, S., Raveendran, M., Manonmani, S., Gopalakrishnan, C., Arul, L., Kokiladevi, E. and Sudhakar, D. 2021. Adenine Base Editor Creates Novel Substitution Mutations in *eIF4G* Gene of Rice. *Madras Agricultural Journal*, **108**(special), 1.

Li, L., Shi, F., Wang, G., Guan, Y., Zhang, Y., Chen, M., Chang, J., Yang, G., He, G., Wang, Y. and Li, Y. 2022. Conservation and divergence of *SQUAMOSA-PROMOTER BINDING PROTEIN-LIKE* (*SPL*) gene family between wheat and rice. *International Journal of Molecular Sciences*, **23(**4): 2099. [Cross Ref]

Liu, Q., Harberd, N. P. and Fu, X. 2016. SQUAMOSA promoter binding protein-like transcription factors: targets for improving cereal grain yield. *Molecular Plant*, **9**(6): 765-767. [Cross Ref]

Liu, W., Xie, X., Ma, X., Li, J., Chen, J. and Liu, Y.-G. 2015. DSDecode: a web-based tool for decoding of sequencing chromatograms for genotyping of targeted mutations. *Molecular plant*, **8**(9): 1431-1433. [Cross Ref]

Lu, Q. and Lu, C. 2004. Photosynthetic pigment composition and photosystem II photochemistry of wheat ears. *Plant Physiology and Biochemistry*, **42**(5): 395-402. [Cross Ref]

Luo, J., Liu, H., Zhou, T., Gu, B., Huang, X., Shangguan, Y., Zhu, J., Li, Y., Zhao, Y., Wang, Y., Zhao, Q., Wang, A., Wang, Z., Sang, T., Wang, Z. and Hana, B. 2013. *An-1* encodes a basic helix-loop-helix protein that regulates awn development, grain size, and grain number in rice. *The Plant Cell*, **25**(9): 3360-3376. [Cross Ref]

Luong, N. H., Balkunde, S. G., Shim, K.-C., Adeva, C., Lee, H.-S., Kim, H.-J. and Ahn, S-Nag. 2022. Characterization of domestication loci associated with awn development in rice. *Rice*, **15**(1): 1-13. [Cross Ref]

Nithya, S., Kumam, Y., Varanavasiappan, S., Kumar, K.K., Muthamilan, M. and Sudhakar, D. 2020. Targeted mutation in *eIF4G* gene in rice. *Electronic Journal of Plant Breeding*, **11**: 1194-1199. [Cross Ref]

Rebetzke, G., Bonnett, D. and Reynolds, M. 2016. Awns reduce grain number to increase grain size and harvestable yield in irrigated and rainfed spring wheat. *Journal of Experimental Botany*, **67**(9): 2573-2586. [Cross Ref]

Takano-Kai, N., Jiang, H., Kubo, T., Sweeney, M., Matsumoto, T., Kanamori, H., Padhukasahasram, B., Carlos Bustamante, C., Atsushi Yoshimura, A., Doi, K. and McCouch, S. 2009. Evolutionary history of *GS3*, a gene conferring grain length in rice. *Genetics*, **182**(4): 1323-1334. [Cross Ref]

Verma, A., Prakash, G., Ranjan, R., Tyagi, A. K. and Agarwal, P. 2021. Silencing of an ubiquitin ligase increases grain width and weight in *indica* rice. *Frontiers in Genetics*, 1650. [Cross Ref]

Wang, L. and Zhang, Q. 2017. Boosting rice yield by fine-tuning *SPL* gene expression. *Trends in Plant Science*, **22**(8): 643-646. [Cross Ref]

Wang, S., Li, S., Liu, Q., Wu, K., Zhang, J., Wang, S., Wang, Y., Chen, X., Zhang, Y., Gao, C., Wang, F., Haixiang, H. and Fu, X. 2015. The *OsSPL16-GW7* regulatory module determines grain shape and simultaneously improves rice yield and grain quality. *Nature genetics*, **47**(8): 949-954. [Cross Ref]

Wang, S., Wu, K., Yuan, Q., Liu, X., Liu, Z., Lin, X., Zeng, R., Zhu, H., Dong, G., Qian, Q., Zhang, G. and Fu, X. 2012. Control of grain size, shape and quality by *OsSPL16* in rice. *Nature genetics*, **44**(8): 950-954. [Cross Ref]

Wang, S.-L., Zhang, Z.-H., Fan, Y.-Y., Huang, D.-R., Yang, Y.-L., Zhuang, J.-Y. and Zhu, Y-.J. 2022. Control of grain weight and size in rice (*Oryza sativa* L.) by *OsPUB3* Encoding a U-Box E3 Ubiquitin Ligase. *Rice*, **15**(1): 1-12. [Cross Ref]

Xie, K., Wu, C. and Xiong, L. 2006. Genomic organization, differential expression, and interaction of SQUAMOSA promoter-binding-like transcription factors and microRNA156 in rice. *Plant physiology*, **142**(1): 280-293. [Cross Ref]

Xie, X., Ma, X., Zhu, Q., Zeng, D., Li, G. and Liu, Y.-G. 2017. CRISPR-GE: a convenient software toolkit for CRISPR-based genome editing. *Molecular plant*, **10**(9): 1246-1249. [Cross Ref]

Yamaguchi, K., Yamamoto, T., Segami, S., Horikawa, M., Chaya, G., Kitano, H., Iwasaki, Y. and Miura, K. 2020. *gw2* mutation increases grain width and culm thickness in rice (*Oryza sativa* L.). *Breeding Science*, **70**(4): 456-461. [Cross Ref]