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IDENTIFICATION OF A NOVEL ALLELE OF THE SD1 GENE IN GUANGXI COMMON WILD RICE Y11 AND THE ACQUISITION OF DWARF PLANTS

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Abstract: To explore the novel function of the sd1 gene in the context of the “Green Revolution”, this study was conducted on Guangxi common wild rice Y11, which is genetically rich. The objective was to investigate its sd1 allele and verify its biological function using a gene editing system. Through genetic analysis, the sd1 allele of Y11 was successfully cloned and compared with those of Nihon Haru and "93-11" to identify allelic differences. It was deduced that the sd1 allele of Y11 is a strong allele. Subsequently, the coding sequence of the sd1 gene was cloned and sequenced to elucidate the gene structure and sequence characteristics. Bioinformatics analysis revealed that the encoded protein possesses specific physicochemical properties and functional domains, and its tertiary structure exhibits a high degree of similarity to those of other varieties. By employing the CRISPR/Cas9 system, the editing vector CRISPR-sd1 was constructed with sd1 as the target gene. Pure sd1 mutants were obtained in the T0 generation of both transformation recipients, yielding plants with reduced height. This study demonstrated that the sd1 gene of Guangxi wild rice Y11 represents a strong allele for plant height that has not been exploited in cultivated rice. Gene editing produced materials with significant alterations in plant height, highlighting the value of utilizing this gene and providing an excellent allele target for future "new strain breeding." Additionally, it furnished a material and theoretical foundation for enhancing the excellent traits of rice.
Keywords: Guangxi Common Wild Rice Y11; Gene clone; Sd1; Gene editing

1 INTRODUCTION

Rice is one of the most important food crops in the world and the largest food crop in China. Its output has been constantly challenging new peaks over the years. During the long domestication process from common wild rice to Asian cultivated rice (Oryza sativa L.), due to long-term artificial screening and genetic bottleneck effects, many excellent alleles were unfortunately lost [1], such as insect-resistant [2] and disease-resistant [3] gene resources. This has led to the homogenization of cultivated rice seeds [4], especially posing a huge challenge to the resistance of cultivated rice to both biotic and abiotic stresses.

The common wild rice (Oryza rufipogon Griff.), known as the "ancestor" of rice, has unfortunately been listed as an endangered species . Wild rice resources have accumulated a large amount of genetic resources that resist biotic and abiotic stresses and excellent quality characteristics [5] in response to the severe challenges of stable and increased rice production in the future, playing an indispensable role. The exploration, protection, development, and utilization of wild rice resources have received increasing attention [6]. It is not only a valuable resource bank for rice breeding but also contains extremely high research, development, and application value. As the "root" of rice, the rich genetic resources of common wild rice are of profound significance for the research on the origin, evolution process, and type classification of rice plants [7]. Through in-depth exploration and utilization of wild rice resources, it is expected to increase the yield of rice, optimize the quality, and enhance the stress resistance, ultimately providing strong technical support for rice production. However, wild rice also faces many challenges in breeding practice, such as the characteristics of difficult husking, small grains, low yield, and easy grain dropping, which are difficult to meet the demands of large-scale planting. In addition, there are problems such as incompatibility of distant hybridization between wild rice and cultivated rice and infertility of hybrid offspring [8], which further restrict the seed quality innovation and breeding efficiency of wild rice. Based on the current research results of wild rice genomics, it provides an important basis for an in-depth analysis of the genetics of complex agronomic traits and the adaptive evolution of wild rice [9].

Constructing core germplasm is the prerequisite and foundation for improving the utilization efficiency of rice germplasm resources, and enhancing the ability of germplasm innovation. At present, the core germplasm of common wild rice in Guangxi [10], local rice seed resources and local varieties has been constructed. With the development of gene editing technology, excellent alleles have broad prospects in the genetic improvement of rice. Attention should be paid to the exploration and utilization of excellent alleles in germplasm resources, which can be approached from two aspects: first, identify and utilize the excellent genes in wild rice and local varieties, and explore their potential in rice breeding through methods such as genomics; second, enhance the application of superior alleles in the creation of new germplasm, and improve breeding efficiency through methods such as developing functional molecular markers and polymeric breeding [11].

The semi-dwarf gene sd1, a cornerstone of the Green Revolution in rice [12]. It has played an important role in the genetic improvement of rice, while the sd1 gene in wild rice has not been studied much. This paper takes the common wild rice Y11 in Guangxi as the research material. Through means such as gene cloning, allele analysis, and construction of gene editing vectors, it deeply explores the function of the new allele sd1, aiming to provide a solid material basis and theoretical foundation for the efficient exploration and utilization of the germplasm resources of common wild rice in Guangxi.

2 MATERIALS

2.1 Test Materials and Reagents

The transgenic receptor material was the common wild rice Y11 of Guangxi (provided by Guangxi Academy of Agricultural Sciences), and all the rice materials were grown in the screen houses with nylon nets of Jiangsu Academy of Agricultural Sciences, with conventional water and fertilizer management. The CRISPR/Cas9 vector pYLCRISPR/Cas9-MTMono dual vector and Escherichia coli DH5α used in this study were preserved in our laboratory.
The primer sequences used in this study are shown in Table 1. The primers were synthesized by Shanghai Shenggong Bioengineering Co., LTD. The sequencing was completed by Shanghai Shenggong Bioengineering Co., LTD. The kits used in the experiment include the Plant Total RNA Extraction Kit (Tiangen Biochemical Technology, Beijing Co., LTD., DP432), the cDNA reverse transcription kit (Beijing Tiangen Biotechnology Co., LTD., KR116), and the DNA gel recovery kit (Beijing Qingke Biotechnology Co., LTD., GE0101). Zero-background pTOPO-TA Simple Cloning Kit (Wuhan Junode Biotechnology Co., LTD., V6002).

2.2 DNA Extraction Test Materials and Reagents

An appropriate amount of young leaves of wild rice was subjected to liquid nitrogen rapid freezing treatment and then ground into powder. The total genomic DNA in these powders was extracted using the CTAB method. The extracted DNA samples need to be detected by a UV spectrophotometer to screen for high-quality samples with an OD260/OD280 ratio ranging from 1.8 to 2.0, and these samples should be stored at -20℃ for subsequent experiments.

2.3 RNA extraction test materials and reagents

80-100mg of Y11 leaves from common wild rice in Guangxi was taken. Total RNA was extracted according to the instructions of the plant tissue RNA Extraction kit. After detection, the optical density ratio of total RNA at OD260 /OD280 was between 1.8 and 2.0. The extracted total RNA was stored at -80℃ for future use. The total RNA extracted was used for the synthesis of the first strand cDNA using the instructions of the reverse transcription kit.

2.4 Cloning of the sd1 Gene

Primers were designed based on the sequence of the Japanese Haru sd1 gene (registration number: LOC_Os01g66100) found on NCBI (Table 1), and highly specific primers were synthesized.
Using the total DNA and total RNA of Y11 common wild rice in Guangxi as templates, the sd1 allele was amplified using SD-G1, SD-G2, SD-C1, SD-C2, and SD-C3 primers. The amplified target fragments were identified by PCR amplification, respectively. The sizes of the amplified products were compared and analyzed by agarose gel electrophoresis technology. Subsequently, the gel area containing the target fragment was cut out, and DNA recovery treatment was carried out on this part of the gel in accordance with the operation guide of the DNA gel recovery kit. The purified PCR products were sequenced and analyzed. After splicing the sequenced fragments, the complete genome sequence and coding region sequence of sd1 of common wild rice Y11 in Guangxi were obtained.

Table 1 Information on primers used in this study

Primer name	Sense primer sequence(5'-3')	Antisense primer sequence（3'-5'）	Product size(bp)	Annealing Temperature(℃)
SD-B	TGCTGAATTCAAACGCGTTTGTGC	AAGGTGTCGCCGATGTTGAT	401	54
SD-C1	TCACACACGCTCTCAACTCACTCC	CAATGTCGTCCACCATCGTTTTA	645	56
SD-C2	CAAAACACACCGACCGAGAT	TCACAAACAATGGAATGCCC	574	53
SD-C3	GCCTCTCCGTTGATGAATGA	GAACAGAAGCCGATTTTTGG	416	52
SD-G1	TCTCAACTCACTCCCGCTCAA	TGGTGGTGCCTAACTCAACTCA	725	60
SD-G2	CCAACAGCCACGTCCAACAAC	GAGAAGCCCAACCCAATGCTG	919	58

2.5 Construction of CRISPR Vectors

Using the CRISPR - GE (http://skl.scau.edu.cn/targetdesign/) online tools for the design of knockout targets [13], on the sd1 gene exon 2, select one specific sgRNA ATCCTCCTCCAGGACGACGTCGG as the gumming point. Driver OsU 6a promoter, according to the target sequence, synthesis of the corresponding target primers, the sequence is as follows: gRT1: ATCCTCCTCCAGGACGACGTGTTTTAGAGCTAGAAAT; OsU6aT1: ACGTCGTCCTGGAGGAGGATCG GCAGCCAAGCCAGCA, sd1 fragment to the CRISPR/Cas9 carrier, the carrier built knockout sequencing identificatio n, save the sequencing correct plasmid for standby. Send the successfully constructed vector to Boyuan Biological Com pany. The receptor material is the common wild rice Y11 from Guangxi for subsequent genetic transformation experime nts.

2.6 Analysis of Target Gene Mutations

Leaves were collected from the transformed plants, and their DNA was extracted using genomic extraction kits. Then, c DNA was extracted and synthesized from the leaves successively using the total RNA extraction kit and the cDNA reve rse transcription kit. Using leaf DNA and cDNA as templates, respectively, and SD-B as the primer, the Cas9 sequence was amplified. The amplified products were separated and purified by agarose gel electrophoresis. Subsequently, the ge l part containing the target fragment was cut off, and the recovery process was carried out in accordance with the operati on manual of the DNA gel recovery kit. The purified PCR products were ligated with the T vector and transferred to D H5α Escherichia coli competent spread plates for 12 hours of culture. The recombinant positive clones were extracted a s plasmids for sequencing analysis to obtain the mutation status of each allele. The differences between the sequencing r esults and the wild-type sequences were compared using the SnapGene software.

2.7 Phenotypic Analysis

After the rice matured, the plant heights of the wild type and the knockout mutant were determined for analysis and mea surement. Gently place the zero-mark end of the hard ruler on the soil surface, lift the entire rice seedling, and read the t op of the highest panicle as the panicle height.

3 RESULTS

3.1 Cloning and Sequence Alignment Analysis of the sd1 Gene

3.1.1 Cloning of the sd1 gene

Guangxi common wild rice (Y11) genomic DNA and cDNA templates were used to amplify the sd1 gene's full-length g enomic and coding sequences. The full-length genomic sequence of sd1 spans 2,742 bp, with a coding sequence (CDS) of 1,170 bp, according to the results of assembling the sequenced fragments and aligning them with the Nipponbare refe rence sequence. Three exons (557 bp, 322 bp, and 291 bp) and two introns (103 bp and 1,472 bp) make up the gene stru cture, which codes for a 389 amino acid protein. The gene structure is illustrated in Figure 1.

Figure 1 Structure of sd1 gene gene of Guangxi Common Wild Rice Y11

3.1.2 Sequence alignment analysis of the sd1 gene

Comparative analysis of the sd1 CDS sequence with allele sequences from publicly sequenced cultivars Nipponbare (ja ponica) and "93-11" (indica) revealed distinct variations (Figure 2). Guangxi common wild rice Y11 exhibited 99.57% coding sequence homology (5 nucleotide differences, 2 amino acid substitutions) and 99.74% genomic sequence homol ogy with Nipponbare, while showing 99.71% coding homology (2 nucleotide differences, no amino acid changes) and 9 9.25% genomic homology with "93-11".

The japonica sd1 allele harbored SNPs across all three exons compared to the wild rice allele: in exon 1, the codon GG G (encoding Gly100) mutated to GAG (Glu); in exon 2, synonymous mutations occurred at positions 171 (GCA → GC C), 190 (GAA → GAG), and 218 (TAT → TAC); in exon 3, the codon CGG (Arg340) was altered to CAG (Gln). In co ntrast, the indica sd1 allele displayed two synonymous mutations in exon 2 (positions 171 and 190) and a nonsense mut ation in exon 3 (position 342), introducing a premature termination codon that abolished gene function. Based on these f indings, the Guangxi wild rice sd1 allele is classified as the strongest functional allele; the japonica allele, impaired by missense mutations (Gly100Glu and Arg340Gln), is a weak functional allele; and the indica allele, with a truncated prot ein due to the premature stop codon, is a non-functional allele.

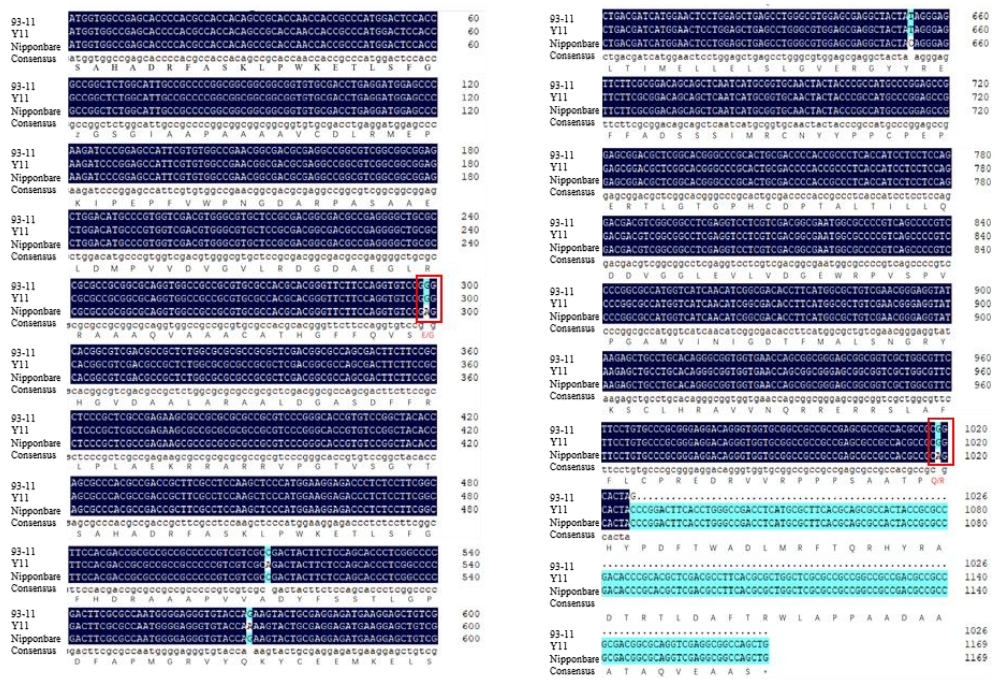


Figure 2 SComparative Analysis of CDS Sequences of sd1 Gene and Protein Sequences of Y11, Nihon Haru, and 93-11 of Common Wild Rice in Guangxi

3.1.3 Bioinformatics analysis of the sd1 gene

The full-length cDNA of the sd1 gene in Guangxi common wild rice (Y11) spans 1,170 bp, encoding a protein of 389 a mino acids. Physicochemical characterization of the SD1 protein using the ExPASy ProtParam tool revealed a molecula r formula of $C_{1881}H_{2907}N_{537}O_{551}S_{19}$, with a theoretical isoelectric point (pI) of 5.96 and a relative molecular mass o f 42.47 kDa. The instability index was calculated as 44.47 (above the threshold of 40), suggesting potential protein insta bility, while the grand average of hydropathicity (GRAVY) value of -0.243 indicated hydrophilic properties.

Secondary structure and functional domain predictions via the SMART database demonstrated that the SD1 protein of Y11 contains five functional domains at amino acid positions 5−16, 26−32, 82−91, 104−115, and 373−388, consistent w ith Nipponbare. In contrast, the "93-11" sd1 allele lacked the 373−388 domain due to a premature termination codon ca used by a nonsense mutation in exon 3.

Tertiary structure modeling using SWISS-MODEL (Figure 3) showed 99.49% structural similarity between Y11 and Ni pponbare and 99.41% similarity between Y11 and "93-11".

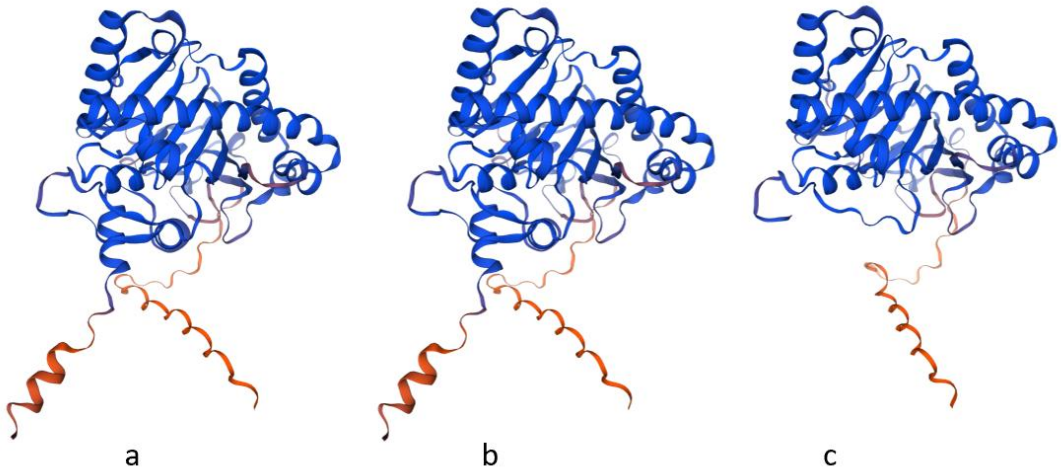


Figure 3 SWISS Website Prediction sd1 Tertiary Structure. (a. Predicted tertiary structure of the sd1 allele in Guangxi common wild rice; b. Predicted tertiary structure of the sd1 allele in Nihon Haru; c. Predicted tertiary structure of the sd1 allele in “93-11”).

3.2 Creation of sd1 Gene-Edited Mutants in Guangxi Common Wild Rice Y11

3.2.1 Construction of the CRISPR/Cas9 gene editing vector

A single highly specific target site adjacent to the PAM sequence was selected on the sd1 gene using CRISPR-GE. The 12 bp sequence flanking the PAM in the reference genome was confirmed to be unique, with only one matching locus, ensuring targeting specificity. A nested PCR strategy was employed to construct the OsU6a-sd1 expression cassette containing this target site, with BsaI restriction sites introduced at both ends. Leveraging the Golden Gate assembly system′s sequence-specific cleavage by BsaI, the expression cassette and the backbone vector pYLCRISPR/Cas9-Pubi-H were digested simultaneously with BsaI. The digested fragments were ligated using T4 DNA ligase, resulting in the successful assembly of the final binary vector pYLCRISPR/Cas9-Pubi-H-sd1 (Figure 4).

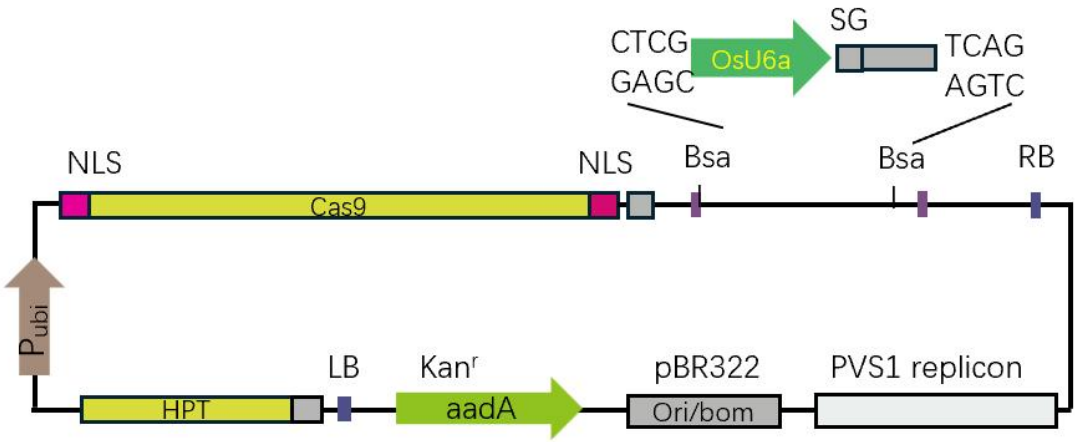
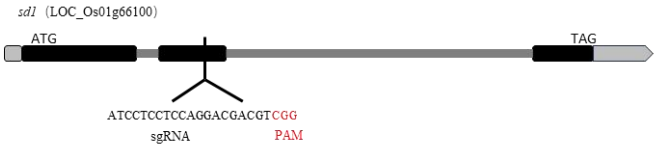


Figure 4 Schematic Diagram of sd1 Target Sites and Knockdown Expression Vectors

3.2.2 Mutation detection and phenotypic analysis of T0 generation transgenic plants

The constructed sd1 knockout expression vector was introduced into Guangxi common wild rice (Oryza rufipogon) Y11 callus via Agrobacterium-mediated genetic transformation, yielding eight T0 generation transgenic seedlings. The DNA fragments containing the target site were amplified using primer SD-B for PCR sequencing. Sequence alignment revealed that heterozygous mutations at the target site were characterized by overlapping peaks (single peaks replaced by double peaks) in chromatograms. The sd1 gene fragments were amplified, cloned into the pTOPO-TA vector, and transformed into E. coli. Positive clones were compared with the wild-type sd1 gene sequence of Guangxi common wild rice Y11. Comparative analysis of PCR sequencing results demonstrated the following mutational profiles: all eight transgenic lines exhibited various types of sequence variations at the target site. As shown in Figure 5, base deletions and insertions were observed in all eight lines, with two lines additionally displaying allelic replacement mutations.



	Target Site	Mutation Type	Culm Height (cm)
WT	ATCCTCCTCCAGGACGACGTCGG	_	125
Y11-Z2-1	ATCCTCCTCCAGGACGACGTCGG ATCTCCTCCAGGACGCTCGTCGG	1-aa deletion Non-mutant	99.43
Y11-Z2-2	ATCCTCCTCCAGGACGACGTCGG ATCCTCCTCCAGGACG TACGTCGG	1-aa deletion Frameshift Mutation	98.29
Y11-Z2-3	ATCCTCCTCCAGGACGACGTCGG ATCCTCCTCCAGGACGTCGTCGG	Frameshift Mutation Non-mutant	66.5
Y11-Z2-4	ATCCTCCTCCAGGACGACGTCGG ATCCTCCTCCAGGACGACGTCGG	Frameshift Mutation Non-mutant	62
Y11-Z2-6	ATCCTCCTCCAGGACGACGTCGG	1-aa deletion	112.43
Y11-Z2-13	ATCCTCCTCCAGGACGACGTCGG ATCCTCCTCCAGGACG AACGTCGG	Frameshift Mutation Frameshift Mutation	61.5
Y11-Z2-14	ATCCTCCTCCAGGACGACGTCGG ATCCTCCTCCAGGACGACGTCGG	Frameshift Mutation 1-aa deletion	107
Y11-Z2-16	ATCCTCCTCCAGGACGACGTCGG	1-aa deletion	93.67

Figure 5 Editing of Rice T0 Generation Transformants with PYLCRISPR/Cas9Pubi-H-sd1 Vector
Note: Red for deletion, green for replacement, orange for insertion.

Guangxi common wild rice (Oryza rufipogon) Y11 exhibits a plant height of approximately 130 cm during the reproductive heading stage and displays a prostrate growth habit. In this study, sd1 was edited to reduce the plant height of Y11. As shown in Figure 5, the sd1 mutants exhibited significantly reduced plant heights ranging from 61.5 - 112 cm compared to the wild type. These results confirm the successful editing of plant height, with lines T0-Z2-3, T0-Z2-4, and T0-Z2-13 showing the most pronounced reduction in plant height compared to wild-type Y11. Sequence analysis revealed th

at base deletions caused frameshift mutations, leading to the loss of protein function due to altered translation. Further g enotypic characterization (Figure 5) demonstrated that lines T0-Z2-1, T0-Z2-2, T0-Z2-3, T0-Z2-4, T0-Z2-13, and T0-Z 2-14 harbored heterozygous mutations, while lines T0-Z2-6 and T0-Z2-16 carried biallelic homozygous mutations(Figu re 6). The latter group showed no segregation in progeny, with phenotypes strictly consistent with their genotypes.

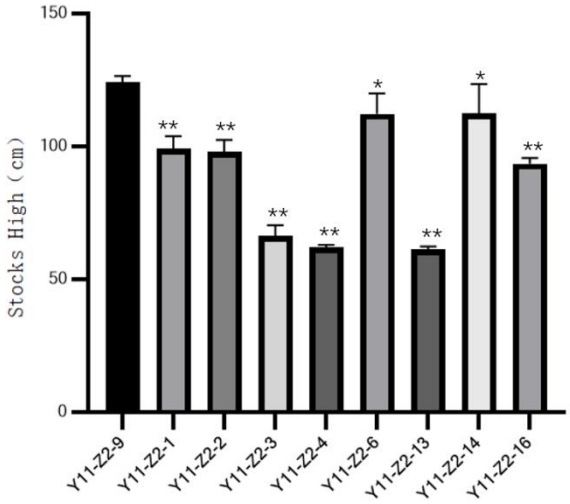


Figure 6 Rice Plant Height of Transgenic Lines in T0 Generation

Note: Plant height in cm, ** represents <0.01 level of significance, * represents <0.05 level of significance

4 DISCUSSION

The sd1 gene in rice, a pivotal gene in the Green Revolution, regulates plant height primarily through the gibberellin bio synthesis pathway, serving as a critical genetic factor influencing rice yield and quality . Since its discovery, it has garn ered significant research attention [14]. Previous studies on sd1 alleles have identified diverse functional variants. For in stance, dwarf cultivars such as Deo-geo-woo-gen carry the sd1-d allele, characterized by a 383 bp deletion at a specific site, which leads to a pronounced dwarf phenotype. Similarly, the sd1-9311 allele in cultivar "93-11" harbors SNPs in e xon 3 and a premature termination codon, resulting in loss of function due to frameshift mutations [15]. The sd1-EQ all ele in the japonica cultivar Nipponbare, a hypofunctional variant, contains SNPs causing amino acid substitutions in spe cific exons. These allelic variations exert distinct effects on plant height and agronomic traits. Loss-of-function alleles, s uch as sd1-d, significantly reduce plant height and enhance lodging resistance. For example, Japan has deployed sd1-d i n certain japonica cultivars, though it remains unused in modern Chinese japonica breeding. However, semi-dwarf japo nica breeding faces challenges, including greater complexity compared to indica rice and reduced genetic diversity due t o the widespread use of sd1 alleles. Notably, sd1-d has been reported to impair fertility under low temperatures and incr ease drought sensitivity [16]. In this study, the sd1 allele of Guangxi common wild rice (Oryza rufipogon) Y11 exhibite d marked differences from these dwarf-associated alleles. Sequence alignment with reference cultivars Nipponbare and "93-11" revealed that while Y11′s sd1 allele contains SNPs, it does not confer the extreme dwarfism observed in sd1-d . Wild rice (Oryza rufipogon), a close wild relative of cultivated rice, represents a reservoir of valuable genetic resources for improving cultivated rice in terms of yield, environmental adaptability, and grain quality. However, reproductive bar riers between wild and cultivated rice—manifested as hybrid sterility or inviable hybrid seeds—severely limit the direct utilization of wild rice alleles in breeding programs [17]. In this study, CRISPR/Cas9-mediated editing of the sd1 gene in Guangxi wild rice Y11 generated homozygous sd1 mutants with reduced plant height in the T0 generation, identifyin g a novel target for plant height modification. This work provides technical insights for leveraging wild rice genetic reso urces and supports "novel plant-type breeding." Previous studies have explored diverse genetic resources and breeding t echnologies to develop high-yielding, stress-resistant, and quality-enhanced rice varieties. Our investigation of Y11′s sd 1 allele adds to this body of knowledge, underscoring the untapped potential of wild rice in modern breeding. Wild rice harbors extensive genetic diversity; by mining and utilizing its superior alleles alongside advanced gene-editing technol ogies, we can accelerate the development of new cultivars tailored to agricultural demands. This aligns with current tren ds in rice breeding, which emphasize the integration of wild relative resources and innovative technologies to enhance b reeding efficiency.

Meanwhile, this paper conducts a detailed analysis of the edited mutants and reveals a close correlation between differe nt mutation types (such as base deletion, insertion, substitution, etc.) and the plant height reduction phenotype. This alig ns with previous studies on the genotype-phenotype association in gene-edited mutants, demonstrating that alterations i n gene sequences directly affect protein function, thereby manifesting in phenotypic changes. For instance, some lines i n this study exhibited frameshift mutations due to base deletions, leading to a loss of protein function during translation and ultimately resulting in significantly reduced plant height. Similar observations have been reported in other gene-edit ing studies, further underscoring the importance of gene-editing technology in elucidating the relationship between gene function and phenotype.

In summary, this study shares similarities with previous research in sd1 gene investigation, gene-editing applications, m utant analysis, and rice breeding, while also presenting unique findings. These results not only enrich the fields of rice g enetics and breeding but also provide critical theoretical foundations and practical insights for future research on rice gr owth and development mechanisms, exploitation of wild rice resources, and cultivation of high-yield, high-quality rice varieties. Additionally, we acknowledge certain limitations in this study, such as the incomplete assessment of other agr onomic traits in the edited mutants. Follow-up studies will further refine these aspects to deepen our understanding of th e role of the sd1 gene in rice growth, development, and breeding.

COMPETING INTERESTS

The authors have no relevant financial or non-financial interests to disclose.

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