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ORIGINAL ARTICLE

Generation of semi-dwarf rice (*Oryza sativa* L.) lines by CRISPR/Cas9-directed mutagenesis of *OsGA20ox2* and proteomic analysis of unveiled changes caused by mutations

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Abstract

Plant height (PH) is one of the most important agronomic traits of rice, as it directly affects the yield potential and lodging resistance. Here, semi-dwarf mutant lines were developed through CRISPR/Cas9-based editing of OsGA20ox2 in an indica rice cultivar. Total 24 independent lines were obtained in T_0 generation with the mean mutation rate of 73.5% including biallelic (29.16%), homozygous (47.91%) and heterozygous (16.66%) mutations, and 16 T-DNA-free lines (50%) were obtained in T_1 generation without off-target effect in four most likely sites. Mutations resulted in a changed amino acid sequence of mutant plants and reduced gibberellins (GA) level and PH (22.2%), flag leaf length (FLL) and increased yield per plant (YPP) (6.0%), while there was no effect on other agronomic traits. Mutants restored their PH to normal by exogenous GA_3 treatment. The expression of the *OsGA20ox2* gene was significantly suppressed in mutant plants, while the expression level was not affected for other GA biosynthesis (OsGA2ox3 and OsGA3ox2) and signaling (D1, GIDI and SLR1) genes. The mutant lines showed decreased cell length and width, abnormal cell elongation, while increased cell numbers in the second internode sections at mature stage. Total 30 protein spots were exercised, and 24 proteins were identified, and results showed that *OsGA20ox2* editing altered protein expression. Five proteins including, glyceraldehyde-3-phosphate dehydrogenase, putative ATP synthase, fructose-bisphosphate aldolase 1, *S*-adenosyl methionine synthetase 1 and gibberellin 20 oxidase 2, were downregulated in dwarf mutant lines which may affect the plant growth. Collectively, our results provide the insights into the role of *OsGA20ox2* in PH and confirmed that CRISPR–Cas9 is a powerful tool to understand the gene functions.

Keywords CRISPR/Cas9 · Rice · Plant height · Gibberellins · Genome editing

Introduction

Rice (*Oryza sativa* L.) is one of the most important food crops in the world, providing food for approximately 21% of the global population. In recent years, with the rapidly growing population and improvement of people's living standards the rice yield, quality and flavor have accordingly become one of the major objectives of breeders and growers (Wang and Li 2008; Xing and Zhang 2010). Plant height (PH) is an important character having role in architecture of plant and adaptability to environment, that are directly linked with the yield potential (Fischer and Stapper 1987; Zhou et al. 2016). Gibberellin (GA) is an important phytohormone in the regulation of seed germination, flowering, leaf shape, leaf expansion, hypocotyl and stem elongation. Generally, plants with a dwarf or semi-dwarf stature are deficient in GA accumulation. Many genes are involved in GA biosynthesis

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and signaling which plays critical roles in brassinosteroid metabolism (Zhang et al. 2014). The semi-dwarf (SD1) allele controls tall phenotype, while the green revolution recessive allele (sd1) controls semi-dwarf PH. After the *SD1* introduction, the rice has greatly achieved the targeted additional worldwide production. The semi-dwarf miracle rice (*Oryza sativa*) cultivar IR8, which enabled dramatic yield increases and helped to avert food shortages in the "Green Revolution", because of a mutation in the *SD1* (OsGA20ox2) gene (Peng et al. 1999; Monna et al. 2002; Sasaki et al. 2002; Spielmeyer et al. 2002a, b; Nagai et al. 2018). SD1 encodes a GA20-oxidase which is involved in GA biosynthesis and its mutants show different degrees of dwarfism which has been associated with GA level. Breeding for semi-dwarf lines is considered an effective parameter to measure the yield and genetic improvement strategy in rice, wheat and many other crops (Ahmad et al. 2018). In 1930, the *sd1* allele for short stature breeding in rice was in progress in Japan (Tanisaka et al. 1994) and the first semi-dwarf rice variety was developed (Khush 1999) and then commonly distributed in whole Asia for the breeding purpose to improve the yield and lodging resistance. *OsGA20ox2* has an open reading frame (ORF) including 2 introns and 3 exons, encoding 389 amino acids and GA20 oxidase-2, which is a key enzyme in the GA synthesis pathway (Monna et al. 2002). The PH is mostly controlled by the molecular mechanism of endogenous phytohormone functions in same manner. Mostly, GAs, comprises of large group of diterpeniod carboxylic acids such as GA_1 and GA_4, exhibits a key role in plant growth and development (Marciniak et al. 2018; Thomas and Hedden 2018). Many researchers have put an extensive effort to achieve semi-dwarf phenotypes in rice by recently developed molecular breeding approaches (Qiao and Zhao 2011; Wu et al. 2017).

Clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9 (CRISPR/Cas9) has been successfully used to improve traits in rice and heritable putative homozygous mutations generated and transmitted according to classical principles of inheritance (Xu et al. 2015). CRISPR/Cas9 has emerged as a versatile technique for precisely targeted genome editing in cellular organisms. Zinc finger nucleases (ZFNs), and transcriptional activator-like effector nuclease (TALEN) technologies were developed earlier than CRISPR/Cas9 (Gaj et al. 2013; Jung et al. 2018), but due to high efficiency, multiplexing capacity, rapidness, high mutation frequency, flexibility and simplicity, the CRISPR/Cas9 technique gained popularity among the researchers. Previous studies showed that CRISPR/Cas9 has been successfully applied in human cells, vertebrates and plants (Qi et al. 2016; Liang et al. 2017; Gupta et al. 2018; Han et al. 2018). This system contains a small piece of pre-designed RNA complementary to the specific targeted DNA sequence, generates blunt ended double strand breaks (DSBs) which subsequently repaired by the error-prone non-homologous end joining (NHEJ) or homology-directed repair (HDR) (Zheng et al. 2016; Chen et al. 2017). CRISPR/Cas9 technology has been successfully used in *Arabidopsis* (Jiang et al. 2013; Li et al. 2013), rice (Cong et al. 2013; Han et al. 2018; Jiang et al. 2013; Mao et al. 2013; Nawaz et al. 2019; Sun et al. 2016; Shan et al. 2013), maize (Liang et al. 2014), wheat (Shan et al. 2013; Wang et al. 2014), sorghum (Jiang et al. 2013), soybean (Jacobs et al. 2015; Li et al. 2015; Sun et al. 2015) and tomato (Brooks et al. 2014). However, there is need to establish CRISPR/Cas9 system that can be used in commercial cultivars for practical use of genome editing in molecular breeding.

In this work, we used CRISPR/Cas9 technology to specifically induce mutagenesis in *OsGA20ox2*. The Jingxi Glutinous Rice (JGR) (an Indica variety) was selected for genetic transformations, which is widely planted in southern parts of China. JGR has excellent grain quality and genetic potential, but the tall stature makes it vulnerable to lodging and resulted in yield reduction. We achieved delicate and earmark mutation of *OsGA20ox2* gene and homozygous T_1 semi-dwarf lines were obtained successfully with significantly decreased PH, low GA level, downregulated function of regulatory proteins and slightly increased yield without changing other quality traits. CRISPR/Cas9 was used to construct an expression vector containing two sgRNAs and transformed by the Agrobacterium-mediated method and large fragment deletion was achieved and *OsGA20ox2* expression level was suppressed in mutant plants without disturbing other GA-associated genes. This study is an example for targeted gene editing in existing paragon verities with potential for commercialization. These results provide an experimental strategy for the rapid generation of various genetic types of materials using the CRISPR/Cas9 system.

Materials and methods

Experimental materials and growth conditions

The seed of JGR variety were collected from the Rice Research Institute of Guangxi University and were grown in the paddy field of Guangxi University and in Hainan, Sanya, Army District Head Quarters field area during normal rice growing seasons and maintained regularly. The pYL CRISPR/Cas9Pubi-H vector (Figure S1), and promoters (OsU6a and OsU6b) (Figure S2) were provided by State Key Laboratory of Conservation and Utilization of Subtropical Agro-Bioresources, South China Agricultural University, Guangzhou, China. pYL CRISPR/Cas9Pubi-H is a plant expression vector with hygromycin selectable marker having a fragment flanked by two *Bsa*I sites which can be used for inserting sgRNA cassette (Ma et al. 2015a, b). The

primers used in the experiment are synthesized by Beijing Genomics Institute (BGI) (Table S1).

Selection of target sites

The sgRNAs was designed by following a previous report (Liang et al. 2016), and two targets in the first exon of *OsGA20ox2* were selected using online tool CRISPR-GE (http://skl.scau.edu.cn/) (Fig. 1). The targeting specificity of target sequences (including PAM) was confirmed by performing a blast in NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) against the rice genome. The difference of at least two bases compared with similar non-target sequences within the PAM region was assured for a potential target sequence. The structures of both sgRNAs (Figure S3) were developed using online tool CRISPR-P (http://crispr.hzau.edu.cn/cgi-bin/CRISPR2/CRISPR).

Construction of sgRNA expression cassette

The sgRNA expression cassette containing two target sites was constructed by overlapping PCR and two pair of primers were used in each reaction in which 2–5 ng of OsU6a and OsU6b were used as templates. For first PCR reaction, U-F and gR-R were used, respectively, and site-specific primers (Pps-R/Pgs-2, Pps-2/Pgs-L) were used in the second overlapping PCR. Finally, both targets were purified using TaKaRa MiniBest DNA Fragment Purification Kit Ver 4.0, according to manufacturer instructions. The oligos used in constructing the sgRNA vectors for *OsGA20ox2* are listed in Supplementary table S1.

Assembly of sgRNA expression cassette into pYL CRISPR/Cas9 binary vector

The sgRNA expression cassette was constructed by inserting synthesized oligos into the *BSa*I site of the vector pYL-CRISPR/Cas9 (I) (Ma et al. 2015b). For cloning, 20 µl restriction–ligation reaction was prepared as: 60 ng of pYL CRISPR/Cas9 plasmid, 10 ng of expression cassette mixture, 10× CutSmart buffer, 10 mmol/l ATP, 10 U *Bsa*I-HF, 35 U T4 DNA ligase, and reactions were incubated for 15 cycles (5 min at 37 °C, 5 min at 10 °C, 5 min at 20 °C, 5 min and 5 min at 37 °C) (Ma et al. 2015b).

Transformation of *E. coli* and verification of positive colonies

The transformation of expression cassette to DH5-alpha was performed according to previous established method (Ma and Liu 2016). Specific primers, SP-L and SP-R (Table S1) were used to detect the required length. The positive colonies confirming the required product length were sent to BGI for sequencing. The bacterial liquid confirming the targets were incubated for 12 h and plasmids were purified using TaKaRa MiniBEST Plasmid Purification Kit Ver. 4.0, according to manufacturer instructions.

Rice transformation

Transformation was carried out by *Agrobacterium tumefaciens*-mediated co-cultivation method, as previously established procedure (Hiei et al. 1994).

Genotyping of T_0 generation and off-target analysis

The genomic DNA of T_0 generation was extracted with the established CTAB method (Xu et al. 2005) and target-specific primers (SD1T1F/R and SD1T2F/R) were designed to amplify the target sites (Table S1). The amplified PCR products were sent to the BGI for sequencing, and results were decoded and analyzed using online analysis tool DSDecode (http://skl.scau.edu.cn/dsdecode/) (Ma et al. 2015a). The multiple amino-acid sequence alignment was performed using Clustal Omerga online tool (https://www.ebi.ac.uk/Tools/msa/clustalo/). The CRISPR-GE online tool (http://skl.scau.edu.cn/) was used to identify the potential off-target sites for target regions and four off-targets for each target were evaluated. The specific primers flanking the potential off-target sites were designed (Tabe S4) and PCR products were sequenced directly.

Screening of T-DNA-free plants and RT-qPCR analysis

The DNA of T_1 mutant plants was also extracted by CTAB method (Xu et al. 2005), and T-DNA-free plants were screened using Cas9 gene-specific primers Cas9-F and Cas9-R (Table S1). Total RNA was extracted from the panicle tissues and leaves of 30 days old rice seedlings using

Fig. 1 Schematic diagram of sgRNA target sites in *OsGA20ox2*. Exons are indicated as black boxes. T1 and T2 represent Target 1 and Target 2, respectively

RNeasy kit (Qiagen) according to the company instructions. RT-qPCR was performed using a Real-Time (Roche) LC480 PCR in a total volume of 10 µl containing 0.3 µl of reverse-transcribed product, 0.08 µM gene-specific primers and 5.0 µl of ChamQ™ Universal SYBR qPCR Master Mix (Vazyme). PCR cycles were programmed as initial denaturation at 95 °C for 5 min, denaturation at 95 °C for 10 s, annealing at 58 °C for 10 s and extension at 72 °C for 10 s with 45 cycles. The RT-qPCR was also performed to analyze whether the *SD1* gene knock out affects the genes related to GA synthesis (*OsGA2ox3* and *GA3ox2*) and signaling (D1, GIDI and SLR1). The rice *Actin* gene was used as internal control and primers used were designed using online tool (https://biodb.swu.edu.cn/qprimerdb/) (Table S1) and expression was calculated as explained previously (Livak and Schmittgen 2001).

Phenotyping and determination of endogenous GA level

The data were recorded for main agronomic traits, such as PH, panicle length (PL), grains per panicle (GPP), seed setting rate (SSR), flag leaf length (FLL), flag leaf width (FLW), root length (RL), number of lateral roots (NLR), grain length (GL), grain width (GW), 1000-grain weight (GW) and yield per plant (YP) were investigated. Endogenous GA levels were measured for both T_0 and T_1 generations as described previously (Li et al. 2011). The measurement results were expressed in µg/kg FW, and standard methods were followed (Suge 1985), with three replicates. The correlation between PH and GA level was calculated by the method of Pearson correlation test (Schober et al. 2018).

Exogenous GA_3 treatment

The seeds were surface sterilized for 10 min in 70% ethanol solution and washed three times with distilled water and soaked in water at 37 °C for 48 h. The germinated seeds were placed on a 96-well PCR plate and placed in an incubator at 28 °C. After 7 days, 10 µM GA_3 aqueous solutions was sprayed on plants and same amount of distilled water was used as a control, and the PH was measured after 20 days of treatment.

Anatomical observation

0.5 cm long transverse and longitudinal cross-sections of second internodes of mature plants of wild JGR and mutant G7-6-1 were stained with crystal violet and calcofluor and standard method was used according to previous research (Peng et al. 2016). Slices were placed on slides in transverse and longitudinal sections, and the results were visualized using stereomicroscope.

Protein extraction and identification

The proteins were extracted from the stem of WT and mutant line, based on previously described method (Cascardo et al. 2001) with some modifications. The proteins were separated through two-dimensional Gel Electrophoresis (2-D GEL) based on pH through isoelectric focusing (IEF). The gels were immersed in fixative solution [methanol (5): ddH_2O (4): acetic acid (1)] for 1 h and 15 min with 0.5 g commasie blue as staining solution for 3 h (Komatsu et al. 1993). The protein patterns were compared by 2-DE and spots were identified by matrix-assisted laser desorption ionization time-of-flight and quadrupole time-of-flight mass spectrometry (MALDI-TOF–TOF-MS). The in-digestion of proteins was performed according to established protocol (Rosenfeld et al. 1992), and protein database search was carried out using mass values of analyzed peptides by Mascot to search the European Bioinformatics Institute (EMBL-EBI), National Center for Biotechnology Information (NCBI) and SwissProt protein database. The STRING (http://string-db.org/) database was used to predict protein–protein interactions (PPI). The interactions were including direct (physical) and indirect (functional) associations.

Data analysis

All data were analyzed using SPSS 16.0 Statistical Software Program and graphs were developed by GraphPad Prism (version 7.0, GraphPad Software Inc., San Diego, CA, USA).

Results

Plasmid construction

The CRISPR/Cas9 expression cassette was generated using the Golden Gate Assembly method (Fig. 2a), and both target sequences were assembled into the intermediate vector pYL CRISPR/Cas9 Pubi-H, by restriction enzyme-ligation. The order of the U3/U6 promoter driven sgRNA cassettes in a binary construct was arbitrary with the following arrangement; LacZ-OsU6a-T1–OsU6b-T2 (Fig. 2d), and both target sequences were confirmed in constructed expression cassette (Figure S4). The overlapping PCR (Figure S5) was used to construct expression cassette and corresponding sizes of the amplified sgRNA cassettes were as follows: OsU6a-sgRNA (T1): 629 bp; OsU6b-sgRNA (T2) = 515 bp (Fig. 2a). After the transformation of expression cassette to DH5α, the positive colonies were detected using the SP-L1 and SP-R primers and sequencing was performed from both directions (Fig. 2b). The sequence of *OsGA20ox2* in WT was

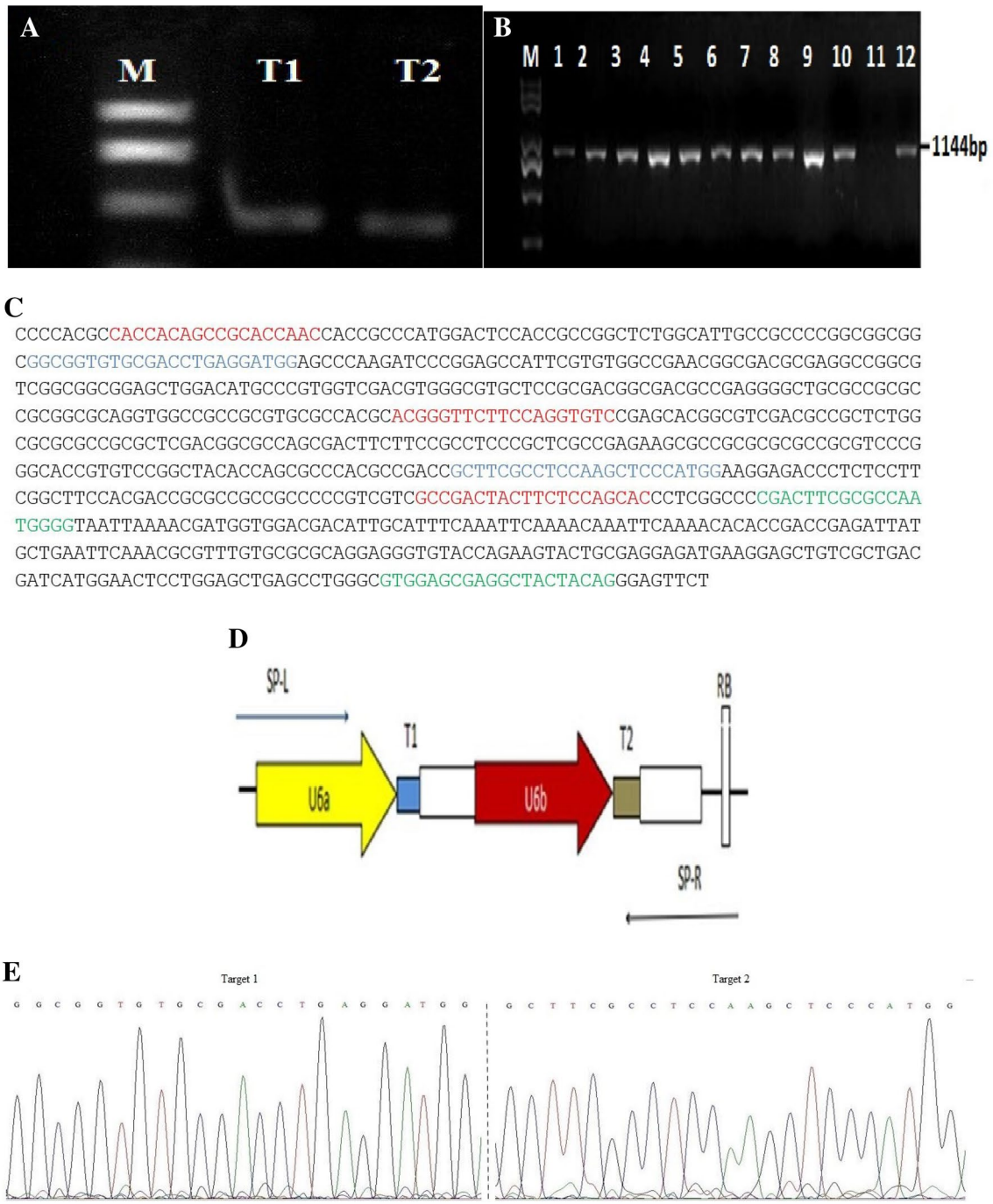


Fig. 2 Expression cassette and sequencing peak map of both targets assembled in pYLCRISPR/Cas9Pubi-H. **a** Gel electrophoresis detection of expression cassette for both targets, *M* DL2000 DNA marker; **b** detection of expression cassettes after transformation of DH5α; *M* DL2000 DNA marker, *1–12* amplified bacterial colonies; 1144 bp: total amplified length of U6a and U6b expression cassette assembly; **c** JGR *SD1* gene sequence of target regions; red sequences represent the upstream and downstream primers of *SD1* partial gene sequence; blue sequences represent the *SD1* gene target sites sequence and position; the sequences in green letters represent the RT-qPCR primers; **d** linking sequence of two expression cassettes U6a-driven Target 1 and U6b-driven Target 2 on pYL CRISPR/Cas9 vector; **e** sequencing peak map of both target sites assembled in pYLCRISPR/Cas9Pubi-H vector

amplified, and specific primers were designed to amplify the target sites (Fig. 2c). Sequencing results confirmed that both targets were assembled in vector successfully (Fig. 2e).

Mutation frequency of T_0 generation and off-target effects

After introducing the construct into rice embryogenic calli

by *Agrobacterium*-mediated transformation, the positive mutant lines using specific hygromycin phosphotransferase primers (HPTF/HPTR), and amplified product was confirmed by gel electrophoresis (Fig. 3a), and finally obtained 24 independent regenerated transgenic lines. The 2 constructs were successfully introduced to rice embryogenic calli by *Agrobacterium*-mediated transformation and total 24 independent mutant lines were obtained in T_0 generation. The mutations were confirmed by sequencing of target regions using target-specific primers. The CRISPR/Cas9 construct that we designed to target *OsGA20ox2* were selected in the first exon (bases 174–193 for Target 1 and 518–537 for Target 2) with high efficiency to achieve expected targeted mutations (Table S2). The sanger sequencing results revealed that there were 4 plants at Target 1 and 6 plants at Target 2 with WT genotype, and the mean mutation rate was 73.5% (Table 1). The mutation rate for SD1T1 was 83.33% of the total number of mutant plants (24 plants) and there were 17 plants with homozygous mutations (70.83%) and only 3 plants showed heterozygous mutations (12.50%). Total 18 mutant plants were achieved on Target 2, with the mutation rate of 75.0% of the total number of mutant plants. Among them, there were seven plants with bi-allelic mutations (29.16%), six plants of homozygous mutations (25%) and only five plants of heterozygous mutations (20.83%). The plants with homozygous mutations were more than heterozygous mutants in both targets and were 14 plants with mutations on both targets (58.33%) (Table 1).

We analyzed the four mutant plants for further investigations and DSDecode analysis of *OsGA20ox2* gene target sites in T_0 generation showed that the mutant line G7-1 showed homozygous mutations with 3 bp deletions and 5 bp insertions on Target 1 and biallelic mutations with 1 bp substitution and deletion on Target 2. Mutant line G7-3 showed indel mutation at Target 1 and homozygous mutation with insertion at Target 2, while mutant line G7-6 showed homozygous

Fig. 3 Detection of positive mutant lines and analysis of mutations. **a** T_0 positive mutant lines; *M* DNA marker, *WT* wild type, 1–19 T_0 transgenic lines; **b** nucleotide sequence alignment and mutation frequency for both target regions. Deletions and insertions are indicated by red dashes and letters, respectively, while yellow highlighted is PAM (protospacer adjacent motif). The numbers on the right side show the sizes of the indels, with "−" and "+" indicating deletion and insertion of the nucleotides

Table 1 Mutation rates of both targets in T_0 genration

Mutation type						
Targets	Bi-allelic	Homozygous	Heterozygous	Chimeric	WT	Total
SD1T1						
NOP	0	17	3	0	4	24
MR	0	70	12	0		83
SD1T2						
NOP	7	6	5	0	6	24
MR	29	25	20	0		74
					Mean MR	73.5

NOP number of plants, *MR* mutation rate (%), *WT* wild type

mutation with the large fragment deletion (137 bp) at Target 1 and (16 bp) at Target 2. Target 1 of mutant line G7-12 showed substitution of 2 bp and homozygous mutation with the insertion of 6 bp at Target 2. The sequence alignment analysis (Fig. 3b) and sequence peak maps (Figure S6) indicated that simultaneous efficient deletions at both target sites of G7-6 within one exon, resulted in a large fragment deletion of 153 bp between the two target sites. The DNA of 24 mutant plants was amplified using specific primers for 12 loci of both targets with highest ranking off-target potential and no secondary off-target mutations were detected from potential off-target analysis (Table S3). The primers used for off-target analysis are enlisted in Table S4. Different types of mutations were observed in T_0 generation (Table S5). The conserved amino-acid sequence of WT and mutant lines were changed (Figure S7).

Endogenous GA level and plant height in T_0 generation

Endogenous GA level were measured for the WT and mutant lines G7-1, G7-3, G7-6 and G7-12. Results clearly showed that all the mutant lines showed decreased PH and GA level and mutant line G7-6 showed minimum PH (114.14 cm) and GA level (1.26 µg/kg Fw), while WT showed maximum PH (143.61 cm) and GA level (1.76 µg/kg Fw). These results also showed that there was a significant positive correlation ($R = 0.99$) between PH and endogenous GA level in WT and mutant lines (Table 2).

T-DNA-free mutants in T_1 generation

Homozygous mutants of T_0 and T_1 were planted and 38 plants were screened to analyze their genetic transformation patterns and thereby detect the presence or absence of the exogenous DNA of the mutant lines. T-DNA-free mutants were selected using cas9-specific detection primers cas9-F/Cas9-R (Table S1). The results showed that 19 mutants were amplified to Cas9 vector sequence with 572 bp fragment and 19 lines were not amplified to the corresponding fragment and thus termed as T-DNA-free plants (Fig. 4a). T-DNA-free strain appeared with a frequency of 50%.

Table 2 Plant height and endogenous gibberellin (GA) content in T_0 mutant lines

T_0 transgenic plant	Mutation type	Plant height (cm)	Content of GA (µg/kg Fw)
JGR	WT	143.61 ± 4.31	1.76 ± 0.11
G7-1	Bi-allelic	121.34 ± 3.22**	1.33 ± 0.14**
G7-3	Homozygous	117.56 ± 2.65**	1.32 ± 0.21**
G7-6	Homozygous	114.14 ± 4.33**	1.26 ± 0.09**
G7-12	Homozygous	119.22 ± 3.64**	1.28 ± 0.06**
Mean		123.17	1.39
		$R = 0.99$, p value = 0.001595	

R Pearson correlation co-efficient

**Significant difference ($p < 0.01$)

Plant height and endogenous GA level in T-DNA-free T_1 mutant lines

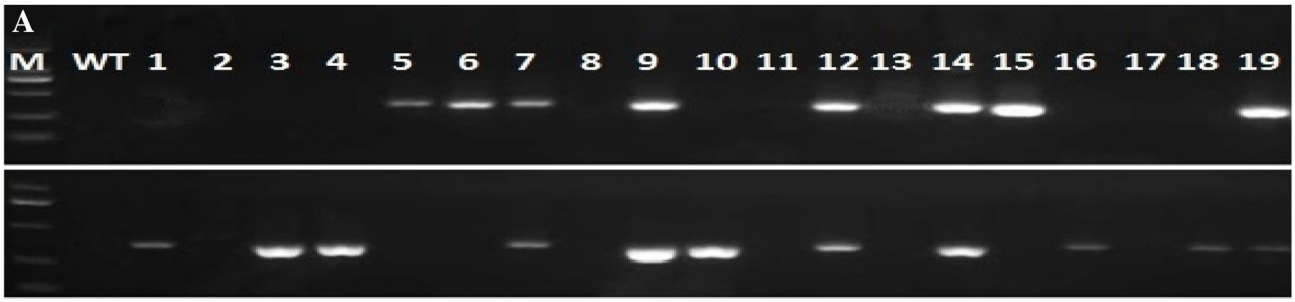
PH and endogenous GA level were measured for the screening of 12 T-DNA-free T_1 lines and compared with T_0 plants at 125 days after germination (DAG). The PH of semi-dwarf mutant lines was decreased (20.52%) from 143.61 to 114.14 cm. The PH and endogenous GA level of each T-DNA-free T_1 lines were positively correlated ($R = 0.30$) (Table 3). T-DNA-free semi-dwarf mutant lines were stably inherited and semi-dwarf plants were obtained with heritable mutations (Fig. 4b).

Performance of agronomic and quality traits in WT and mutant lines

The main agronomic characters of WT and mutant lines were recorded at 125 DAG. The mean results for morphological traits showed that there were no significant differences among all agronomic traits except PH and flag leaf length between mutant lines and WT. The mutant plants showed slightly shorter panicle length, shorter flag leaf length, while there was no any difference in panicle numbers, flag leaf width, grain number per panicle, seed setting rate, and 1000-grain weight, yield per plant, grain length and grain width (Table 4). However, the grain shape and appearance were not affected (Figure S8A–C). Although each internode of G7-6-1 was shortened, the basal internodes including the third, fourth, and fifth internodes (counting from the top) exhibited a higher percentage of length reduction in G7-6-1 (Figure S8E). The yield per plant was increased 6.4% from 15.7 to 16.7 g (Table 4).

SD1 expression level

Total RNA was extracted, and RT-qPCR was used to detect the relative expression of *SD1* gene in WT and mutant plants. Rice *Actin* gene was used as a reference to normalize *SD1* expression between samples. The expression of WT was not altered, and the expression of mutant plants was substantially downregulated in the mutants compared with WT ($p < 0.01$, Fig. 5) indicating that mutations have successfully affected the target gene expression.



B

Generation	Genotype	Target 1	Mutation Type	Target 2	Mutation Type
	WT	CGGCGGCGGTGTGCGACCTGAGGATGGAGCCC		GACCGCTTCGCCTCCAAGCTCCCATGGAAGG	
T_1	G7-1-3	CGGCGGCGGTGTGCGACCTGA---CTATATGGAGCCC	-3,+5	GACCGCTTCGCCTCCA-TGCTCCCATGGAAGG	-1,+1
				GACCGCTTCGCCTCCAAGCTC-CATGGAAGG	-1
T_2	G7-1-3	CGGCGGCGGTGTGCGACCTGA---CTATATGGAGCCC	-3,+5	GACCGCTTCGCCTCCA-TGCTCCCATGGAAGG	-1,+1
				GACCGCTTCGCCTCCAAGCTC-CATGGAAGG	-1
T_1	G7-3-1	CGGCGGCGGTGTGCGACCTG-GGGATGGAGCCC	-1,+1	GACCGCTTCGCCTCCAAGCTCACCATGGAAGG	+1
T_2	G7-3-1	CGGCGGCGGTGTGCGACCTG-GGGATGGAGCCC		GACCGCTTCGCCTCCAAGCTCACCATGGAAGG	+1
T_1	G7-6-3	CGGCGGCGG----------	-137	GACCGC----------CATGGAAGG	-16
T_2	G7-6-3	CGGCGGCGG----------		GACCGC----------CATGGAAGG	-16
T_1	G7-12-2	CGGCGGCGGTGTGCGACCTGA--CCATGGAGCCC	-2,+2	GACCGCTTCGCCTCCCATGCTAAGCTCCCATGGAAGG	+6
T_2	G7-12-2	CGGCGGCGGTGTGCGACCTGA--CCATGGAGCCC	-2,+2	GACCGCTTCGCCTCCCATGCTAAGCTCCCATGGAAGG	+6

Fig. 4 **a** Detection of T-DNA-free mutant lines; *M* DL2000 DNA marker, *WT* wild type. **b** Sequence alignment for transmission of mutations at both target sites in T_1 and subsequent T_2 generations. The targeted sequence is shown in green background and the PAM sequence in gray background. Insertion is represented by red letters, and deletion by red hyphens

Table 3 Plant height and endogenous GA levels in wild-type and T-DNA-free T_1 mutant plants

Genotypes	PH (cm)	GA (μg/kg Fw)
WT	145.42 ± 3.93	1.82 ± 0.2
G7-1-3	120.32 ± 2.55**	1.22 ± 0.13**
G7-1-5	122.65 ± 4.11**	1.25 ± 0.12**
G7-1-8	119.63 ± 2.52**	1.24 ± 0.42**
G7-3-1	116.74 ± 3.18**	1.22 ± 0.31**
G7-3-6	119.62 ± 1.92**	1.23 ± 0.12**
G7-3-9	118.25 ± 2.63**	1.21 ± 0.24**
G7-6-3	115.35 ± 3.25**	1.19 ± 0.13**
G7-6-4	113.15 ± 3.79**	1.15 ± 0.13**
G7-6-7	114.26 ± 2.95**	1.18 ± 0.08**
G7-12-2	118.63 ± 3.42**	1.20 ± 0.16**
G7-12-7	119.53 ± 4.14**	1.21 ± 0.11**
G7-12-9	121.21 ± 2.43**	1.22 ± 0.09**
Mean	111.04	1.26
	$R = 0.97$, p value = 0.00001	

Data are the mean of three replicates

WT wild type, *PH* plant height, *GA* gibberellins, *R* Pearson correlation co-efficient

**Significant difference ($p < 0.01$)

Expression analysis of GA biosynthesis and signaling related genes

Expression analysis of GA-associated gene was performed to analyze whether the mutations in *SD1* mutants affect other GA biosynthesis or signaling genes. The RNA was extracted from rice panicle tissues to evaluate expression level of these genes. The results showed that the expression of GA biosynthesis genes (*OsGA2ox3* and *GA3ox2*) was the same in both mutant line (G7-6-1) and wild JGR. The expression level of signaling pathway-associated genes (D1, GIDI and SLR1) in wild JGR and mutant lines was also similar (Fig. 6). It shows that *SD1* gene editing have no effect on other GA synthesis and signaling genes.

Effect of exogenous GA_3 on mutant plants and wild JGR

To study the effect of exogenous GA treatment on the WT and mutant plants, we applied GA_3 to rice seedlings and their response was analyzed. The PH between control and GA_3-treated seedlings of rice was compared after 25 days and data for PH were recorded. The response of mutant plants to exogenous GA_3 was significant, and their PH was

Table 4 Agronomic traits of the high stalk and semi-dwarf mutants and their wild type

Genotypes	PN	PL (cm)	FLL (cm)	FLW (cm)	GNPP	SSR (%)	GW (g)	YPP (g)	GL (mm)	GWD (mm)
WT	5.9 ± 0.8	28.6 ± 2.5	47.0 ± 4.6	1.6 ± 0.1	135 ± 12	88.4 ± 8.9	24.9 ± 5.3	15.7 ± 2.6	2.92 ± 0.1	2.43 ± 0.2
G7-1-3	6.0 ± 0.4^{ns}	25.4 ± 1.1^{ns}	39.6 ± 2.1*	1.7 ± 0.4^{ns}	138 ± 10^{ns}	89.1 ± 7.2^{ns}	25.0 ± 3.8^{ns}	16.3 ± 2.3^{ns}	2.96 ± 0.1^{ns}	2.40 ± 0.6^{ns}
G7-1-5	5.8 ± 0.2^{ns}	24.2 ± 2.7^{ns}	34.4 ± 3.2*	1.6 ± 0.2^{ns}	136 ± 11^{ns}	88.8 ± 8.3^{ns}	25.7 ± 6.1^{ns}	16.2 ± 2.2^{ns}	2.95 ± 0.4^{ns}	2.41 ± 0.4^{ns}
G7-1-8	5.6 ± 0.1^{ns}	26.0 ± 3.5^{ns}	36.1 ± 2.9*	1.6 ± 0.1^{ns}	132 ± 9^{ns}	88.0 ± 5.6^{ns}	25.2 ± 4.2^{ns}	15.9 ± 1.4^{ns}	2.91 ± 0.4^{ns}	2.45 ± 0.4^{ns}
G7-3-1	6.0 ± 0.4^{ns}	23.5 ± 2.6^{ns}	35.7 ± 2.4*	1.7 ± 0.3^{ns}	130 ± 13^{ns}	87.5 ± 4.5^{ns}	25.8 ± 3.5^{ns}	16.1 ± 1.1^{ns}	2.92 ± 0.2^{ns}	2.45 ± 0.7^{ns}
G7-3-6	6.3 ± 0.7^{ns}	25.3 ± 2.7^{ns}	34.4 ± 3.2*	1.7 ± 0.2^{ns}	126 ± 14^{ns}	88.2 ± 4.2^{ns}	26.4 ± 4.9^{ns}	15.9 ± 2.4^{ns}	2.93 ± 0.2^{ns}	2.45 ± 0.6^{ns}
G7-3-9	6.4 ± 0.3^{ns}	25.0 ± 2.9^{ns}	35.5 ± 3.6*	1.8 ± 0.5^{ns}	129 ± 13^{ns}	88.1 ± 3.6^{ns}	24.9 ± 2.7^{ns}	16.2 ± 3.2^{ns}	2.96 ± 0.3^{ns}	2.42 ± 0.3^{ns}
G7-6-3	6.6 ± 0.5^{ns}	23.8 ± 3.1^{ns}	37.3 ± 1.8*	1.6 ± 0.4^{ns}	131 ± 16^{ns}	87.9 ± 4.2^{ns}	26.1 ± 5.1^{ns}	16.7 ± 2.7^{ns}	2.91 ± 0.3^{ns}	2.44 ± 0.2^{ns}
G7-6-4	6.5 ± 0.6^{ns}	24.4 ± 2.5^{ns}	37.9 ± 3.9*	1.6 ± 0.3^{ns}	134 ± 13^{ns}	88.8 ± 3.5^{ns}	26.3 ± 1.6^{ns}	16.6 ± 2.3^{ns}	2.93 ± 0.1^{ns}	2.43 ± 0.4^{ns}
G7-6-7	6.6 ± 0.1^{ns}	25.8 ± 1.2^{ns}	35.5 ± 4.1*	1.7 ± 0.6^{ns}	130 ± 12^{ns}	87.3 ± 5.9^{ns}	26.9 ± 3.2^{ns}	16.7 ± 2.5^{ns}	2.94 ± 0.5^{ns}	2.43 ± 0.1^{ns}
G7-12-2	6.4 ± 0.4^{ns}	26.6 ± 1.3^{ns}	38.2 ± 2.1*	1.8 ± 0.4^{ns}	129 ± 14^{ns}	87.6 ± 3.4^{ns}	25.5 ± 2.2^{ns}	16.4 ± 1.5^{ns}	2.93 ± 0.1^{ns}	2.44 ± 0.6^{ns}
G7-12-7	5.8 ± 0.2^{ns}	25.0 ± 1.6^{ns}	38.9 ± 3.1*	1.7 ± 0.3^{ns}	126 ± 16^{ns}	88.7 ± 2.5^{ns}	25.2 ± 2.9^{ns}	16.5 ± 2.4^{ns}	2.91 ± 0.4^{ns}	2.47 ± 0.4^{ns}
G7-12-9	6.0 ± 0.3^{ns}	24.5 ± 1.7^{ns}	37.6 ± 2.9*	1.9 ± 0.2^{ns}	127 ± 15^{ns}	87.6 ± 3.1^{ns}	25.9 ± 2.7^{ns}	16.3 ± 0.3^{ns}	2.72 ± 0.6^{ns}	2.46 ± 0.3^{ns}

Data are the mean of five replicates

WT wild type, *PN* panicle numbers, *PL* panicle length, *FLL* flag leaf length, *FLW* flag leaf width, *GNPP* grain number per panicle, *SSR* seed setting rate, *GW* 1000-grain weight, *YPP* yield per plant, *GL* grain length, *GWD* grain width

*Significant difference and ^{ns}represents no significant difference ($p < 0.01$)

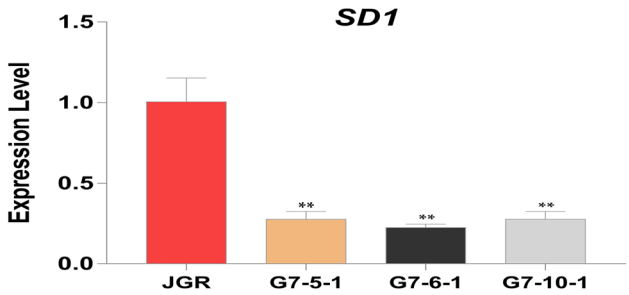


Fig. 5 *SD1* expression level of wild type (JGR) and T_1 mutant plants by RT-qPCR analysis. Data are the mean ± SD of three independent PCR values

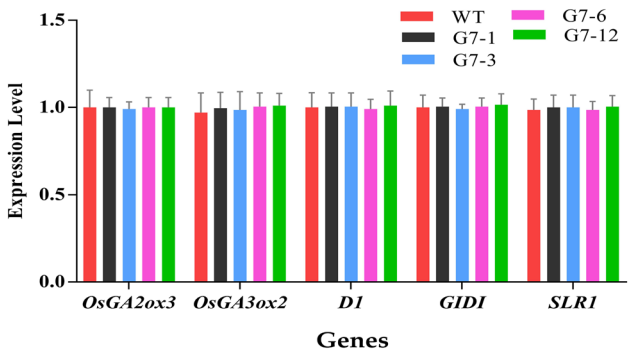


Fig. 6 Expression analysis of GA biosynthesis genes (OsGA2ox3 and GA3ox2) and signaling genes (*D1*, *GIDI* and *SLR1*) in panicle tissues of wild type and mutant lines. The data are the mean ± SD of three independent values

restored identical to that of the wild JGR (Fig. 7a, b). The differences in PH between the mutant plants in controlled condition and exogenous GA_3 treated was significant. The mutant line G7-6-1 showed minimum PH (15.27 cm) in controlled conditions, while the PH of wild-type JGR (25.25 cm) was higher than the mutant plants. Under GA_3 treatment, the mutant lines G7-6-1, G7-5-1 and G7-10-1 restored their PH (31.14 cm, 30.82 cm and 31.38 cm, respectively), nearly equal to the wild JGR (30.99 cm). GA_3 treatment stimulated the growth of dwarf mutants and WT (Fig. 7).

Anatomical observation of second internodal cells

Observations of the cross-section of the second internode of stem indicated that the cortical fiber of G7-6-1 mutant cell was thinner than WT. The staining was clearly observed in small vascular bundles. From the outside to the inside of cross-section of WT consists of thick-walled cells. The vascular bundles were well arranged, regularly rectangular and evenly distributed between parenchyma cells to form longitudinal cell rows (Fig. 8a–d). G7-6-1 mutant cross-section showed thin-walled cells, irregularly shaped and smaller than the wild JGR and the development was immature, but the number of cell layers were increased (Fig. 8a, b). The vascular bundles of mutant plant were scattered among the parenchyma cells and were not developed normally. The results revealed that the development of the stalk of mutant line (G7-6-1) was affected. The cells of WT and mutant line were observed in longitudinal paraffin section. The number of cells of mutant line was increased as compared with the

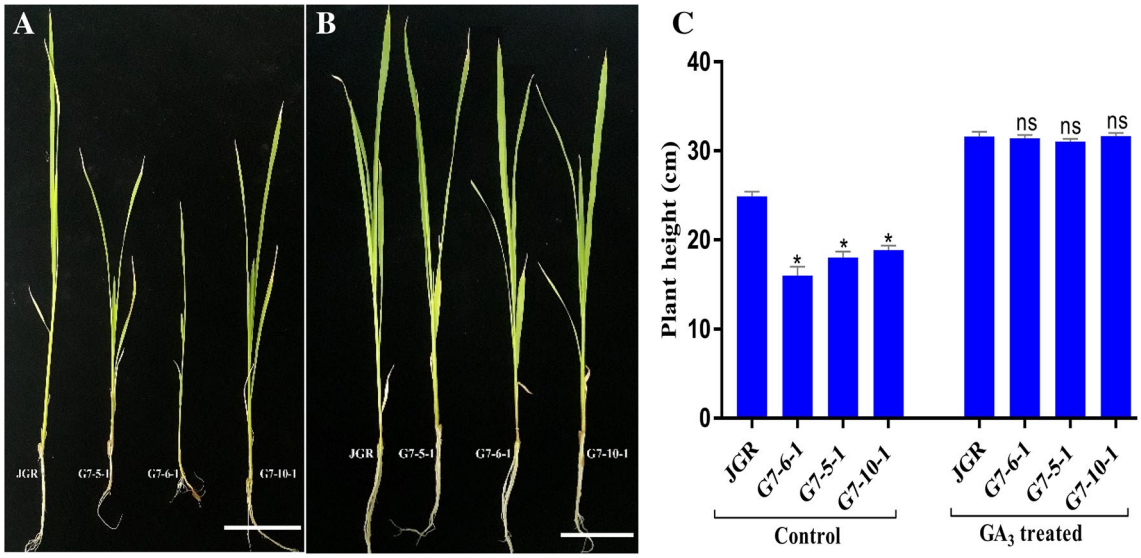


Fig. 7 Seedling phenotype of wild JGR and mutant plants under controlled conditions and GA_3 treatment **a** plants under controlled conditions, **b** plants treated with 10 µM GA_3, **c** plant height of control and GA_3-treated seedling

wild type, but the cell length, size and width were significantly smaller (Fig. 8e–h). The cell numbers of mutant line per unit area were increased 1.31 times (Fig. 8e); the length of mutant cells was 30% decreased than WT (Fig. 8f); the cell size was decreased to 40.84% (Fig. 8g), and the width was reduced two times in mutant line than WT (Fig. 8h).

Protein identification

The SDS-PAGE pattern of proteins showed a large variety of proteins, ranging from 10 to 180 kDa (Figure S9). The stem proteins of WT and mutant line were separated by 2-DE and 30 differential spots were exercised by MALDI-TOF–TOF-MS (Figure S10a, b). A total of 24 proteins were identified, among them 5 protein spots (5, 7, 21, 23 and 27) were down-regulated in G7-6-1 (Table 5). The identified proteins were regulatory and functional proteins having different functions as predicted by European Bioinformatics Institute (EMBL-EBI) and literature.

Protein networks of the identified proteins

To investigate the transduction of mutation, the identified proteins of WT and G7-6-1 were further analyzed using the String 10.5 database (https://string-db.org/) (Szklarczyk et al. 2014). Protein–protein interactions with confidence scores were shown (Fig. 9). The nodes represent the proteins, and the line colors between the nodes indicate protein–protein interaction modes (Fig. 9). Higher co-expression was found between aldehyde dehydrogenase *ALDH2b* and glutamate dehydrogenase (*GDH1*) and maximum proteins showed medium confidence of co-expression (Table 6).

Discussion

Targeted gene mutation to generate DSBs at a predefined genomic location using CRISPR/Cas9 is popular in molecular biology and homozygous mutants are highly desirable in modern breeding. CRISPR/Cas9 is guided by sgRNA followed by PAM, which is recognized by Cas9 coding sequence forming Cas9/sgRNA complex to induce mutations (Li et al. 2016; Sauer et al. 2016; Chen et al. 2017). CRISPR/Cas9 genome engineering tool also used to understand expression and interactions between different genes by generating loss of function of genes. Mutants are important source to study genes related to breeding strategies for crop improvement and many agronomic characters have been improved in rice varieties (Hsu et al. 2013; Xie et al. 2014). Proteomic study is playing an increasingly important role in the functional genomics era and at present, the change at the protein expression level caused by gene mutation is one of the most important parts in proteome studies (Han et al. 2018). Semi-dwarfism is an important trait to increase grain production and improve lodging resistance. Research has proved that bioactive GA played an important role in rice stem elongation and editing the *SD1* gene reduced the PH by affecting the GA biosynthesis in the plant (Hedden, 2003). Loss-of-function mutations of the OsGA20ox2 gene (sd1) cause semi-dwarfism, resulting in lodging resistance and increased grain yields as is exemplified in an indica cultivar IR8 (Sasaki et al. 2002). Since many years, scientists have tried to create semi-dwarfism phenotype in rice by genetic and biotechnological approaches. However, recent advances in plant genome analyses and plant biotechnology make it possible to raise a second green revolution through

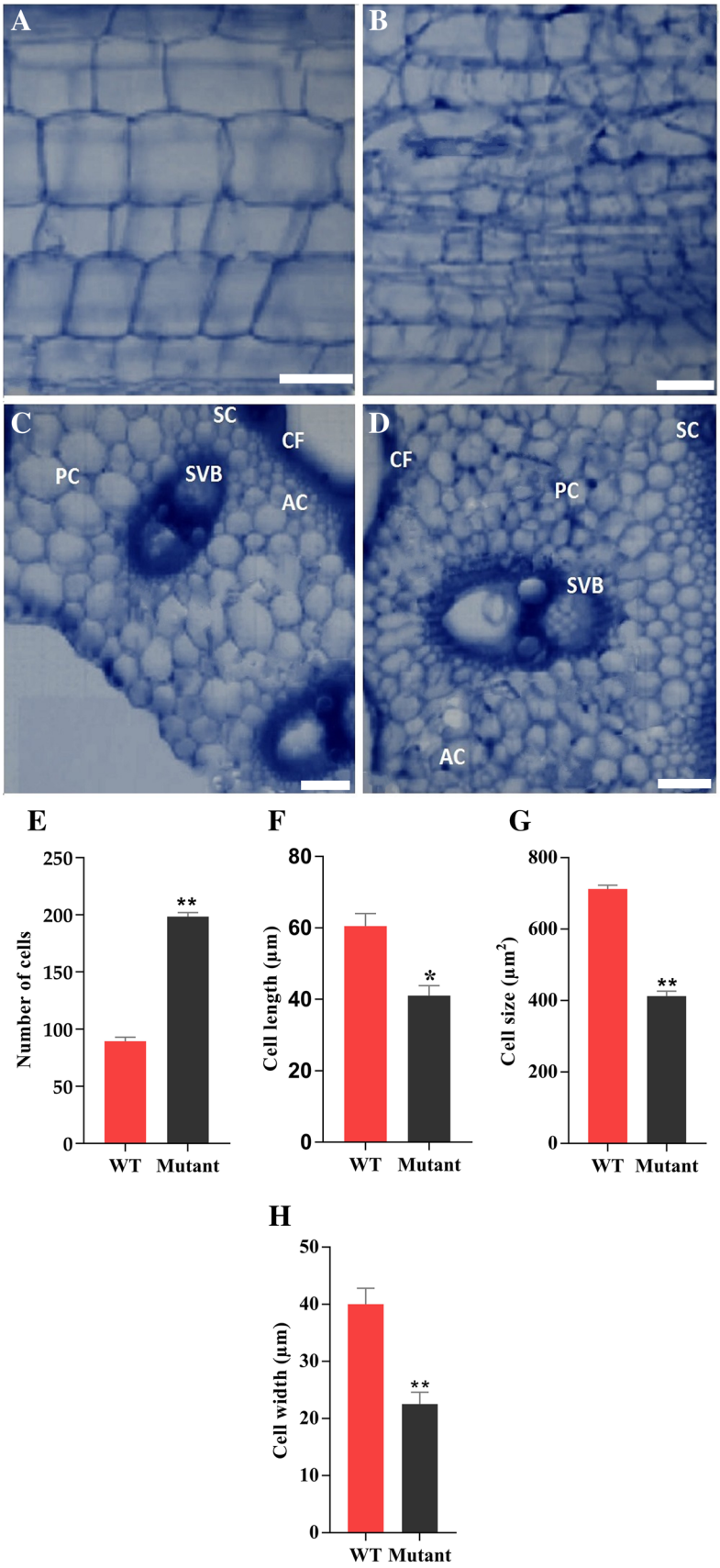


Fig. 8 Anatomical analysis of second internodal cells of wild JGR and G7-6-1. **a** Mutant plant (G7-6-1) second internode longitudinal cell length, **b** wild JGR second intermodal longitudinal cell length, **c** mutant line G7-6-1s internode transverse section of cell and **d** wild JGR second intermodal transverse section of the cell, **e** number of cells in wild type and the mutant line in the same area of longitudinal section, **f** cell length of the wild type and the mutant line in longitudinal section, **g** comparison of the cells size of the wild type and the mutant line in longitudinal section, **h** cell width of the wild type and the mutant line in longitudinal section. *CF* cortical fiber, *PC* parenchyma cells, *AC* aerenchyma, *SVB* small vascular bundle, *SC* sclerenchyma cells. Bars 50 µm

Table 5 Identification of the differentially expressed proteins in wild type and mutant line G7-6-1

Spot no.	Protein description	Accession number	Mr (Da)/pI	Peptide matched	Spot regulation WT	G7-6-1
5	Glyceraldehyde-3-phosphate dehydrogenase, cytosolic	A2YQT7	36413/6.51	8	↑	↓
17	Pyruvate dehydrogenase E1 component subunit beta-1, mitochondrial	Q6Z1G7	39945/5.32	5	↑	↑
25	Aldehyde dehydrogenase ALDH2b	Q9FRX7	59307/6.48	4	↑	↑
14	Glucose-1-phosphate adenylyltransferase small subunit 1, chloroplastic/amyloplastic	Q69T99	54845/6.39	6	↑	↑
12	Glutamate dehydrogenase 1, mitochondrial	Q852M0	44278/6.45	8	↑	↑
18	ATP synthase subunit alpha, mitochondrial	P0C522	55245/5.79	6	↑	↑
7	Putative ATP synthase	Q6ZG90	27145/6.63	3	↑	↓
19	Abscisic stress-ripening protein 5	A2ZBV1	14563/6.19	4	↑	↑
9	Lactoylglutathione lyase	Q948T6	32136/5.53	5	↑	↑
4	Actin-1	A2XLF2	40345/5.29	8	↑	↑
6	Probable photosystem II oxygen-evolving complex protein 2 precursor	Q8GTK4	25934/8.56	9	↑	↑
24	UIpI protease-like proteins	Q69P93	9351/4.97	7	↑	↑
13	Ricin B-like lectin	Q10M12	37542/6.56	4	↑	↑
11	Putative glyceraldehyde 3-phosphate de-hydrogenase	Q9SNK3	46958/6.42	3	↑	↑
21	Fructose-bisphosphate aldolase 1, cytoplasmic	P17784	38543/6.66	6	↑	↓
13	Putative caffeoyl-coA *O*-methyltransferase	Q9XJ19	28432/5.23	7	↑	↑
29	UDP-glucuronic acid decarboxylase	Q8W3J0	39211/7.28	8	↑	↑
7	Putative isoflavone reductase	Q9FTN5	32,856/6.36	9	↑	↑
23	*S*-Adenosyl methionine synthetase 1	A2Y053	43,103/5.29	4	↑	↓
20	Putative ATP synthase beta chain, mitochondrial	Q01859	58,765/5.56	6	↑	↑
2	Gibberellin 20 oxidase 1	P93771	42,189/6.45	7	↑	↑
27	Gibberellin 20 oxidase 2	Q0JH50	42,216/5.32	5	↑	↓
15	Gibberellin 2-beta-dioxygenase 3	Q8S0S6	34,915/6.15	8	↑	↑
10	Aminotransferase-like	Q69ML5	19,251/4.89	7	↑	↑

WT wild type, *ATP* adenosine triphosphate, *UDP* uridine 5′-diphospho-glucuronosyltransferase, *Mr* calculated relative molecular mass of the matched peptide, *Da* Dalton, *pI* isoelectric point

↑ and ↓ represents upregulated and downregulated proteins

the genetic engineering of crops. The knockout mutant in the indica c.v. Kasalath background, which has a complete *OsGA20ox2*, was severely dwarfed. In contrast, knockout mutants in the japonica c.v. Wuyungeng background, which carries an allele that encodes an enzyme with less activity and showed semi-dwarfism and partly infertile phenotype (Asano et al. 2011).

In present work, we explored the high efficiency of CRISPR/Cas9 technology and produced large fragment deletions in *OsGA20ox2* of indica rice variety. Two targets were designed to generate mutations and *Agrobacterium*-based transformation was performed. Semi-dwarf mutants were developed with heritable homozygous mutation efficiencies in subsequent generations, and T-DNA free mutants were screened in T_1 generation. The dwarfing effect of the lines resulted in increase of the yield potential of mutant lines by an average of 6% (Table 4). The mean mutation frequency obtained was 73.5%, and most likely off-target sequences were analyzed and there were no off-target mutations detected for both targets (Table S3). The 19 homozygous T-DNA-free plants were screened from 38 T_1 plants which showed simultaneous homozygous mutations were stably transmitted in T_1 and subsequent T_2 generations. Our results also showed that the lower expression level of *SD1* in dwarf mutant plants disrupted GA synthesis and second internodal cells without affecting the rice grain yield and quality. Studies for semi-dwarf mutants showed that endogenous GA plays key role in PH and modification in GA-associated genes resulted in reduced PH (Marciniak et al. 2018). Different natural mutants of *OsGA20ox2*, have been studied and are generally semi-dwarfed (Asano et al. 2011).

We found that the targeted editing of *SD1* genes does not affect that expression level of other GA synthesis gene

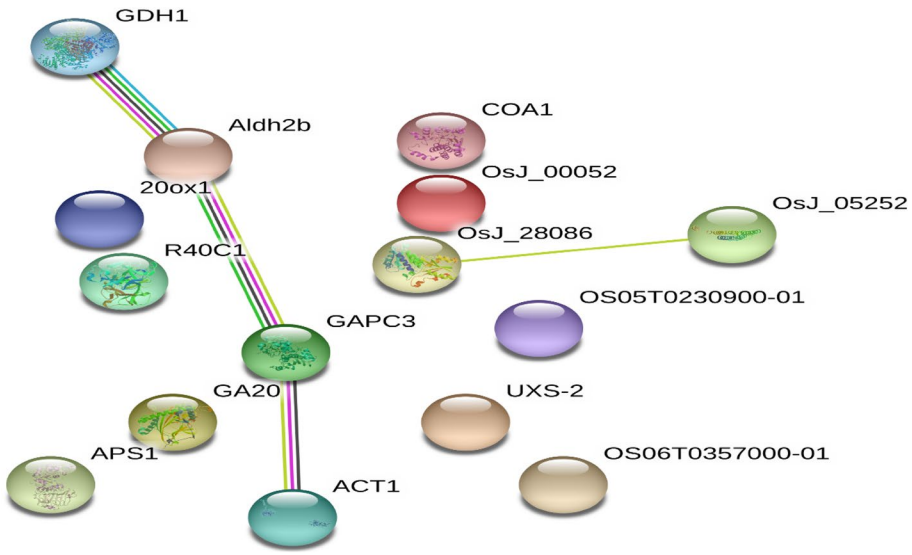


Fig. 9 PPI (protein to protein interaction) visualization by Confidence between proteins. Node represents differential expressed protein, and edge represents interaction relationship between two nodes. A thicker line indicates a stronger interaction between the two proteins. Different colors indicate different evidences i.e. red line—indicates the presence of fusion evidence, green line—neighborhood evidence, blue line—cooccurrence evidence, purple line—experimental evidence, yellow line—textmining evidence, light blue line—database evidence and Black line—represents co-expression evidence

Table 6 Protein to protein interaction (PPI) and confidence score of identified proteins from wild type (WT) and mutant line

Node1	Node2	Node1 accession	Node2 accession	Node1 annotation	Node2 annotation	Score
ACT1	*GAPC3*	OS03T0718100-01	OS02T0601300-01	*Actin-1*	*Glyceraldehyde-3-phosphate dehydrogenase 3, cytosolic*	0.625
Aldh2b	*GAPC3*	OS06T0270900-01	OS02T0601300-01	*Aldehyde dehydrogenase ALDH2b*	*Glyceraldehyde-3-phosphate dehydrogenase 3, cytosolic*	0.449
Aldh2b	*GDH1*	OS06T0270900-01	OS03T0794500-01	*Aldehyde dehydrogenase ALDH2b*	*Glutamate dehydrogenase*	0.701
GAPC3	*ACT1*	OS02T0601300-01	OS03T0718100-01	*Glyceraldehyde-3-phosphate dehydrogenase 3, cytosolic*	*Actin-1*	0.625
GAPC3	*Aldh2b*	OS02T0601300-01	OS06T0270900-01	*Glyceraldehyde-3-phosphate dehydrogenase 3, cytosolic*	*Aldehyde dehydrogenase ALDH2b*	0.449
GDH1	*Aldh2b*	OS03T0794500-01	OS06T0270900-01	*Glutamate dehydrogenase*	*Aldehyde dehydrogenase ALDH2b*	0.701
OsJ_05252	*OsJ_28086*	OS02T0131300-01	OS08T0536000-01	*Os02g0131300 protein; Putative ATP synthase*	*Pyruvate dehydrogenase E1 component subunit beta-1, mitochondrial*	0.453
OsJ_28086	*OsJ_05252*	OS08T0536000-01	OS02T0131300-01	*Pyruvate dehydrogenase E1 component subunit beta-1, mitochondrial*	*Os02g0131300 protein; Putative ATP synthase*	0.453

0.4–0.6 indicates medium confidence, 0.7 indicates high confidence, and 0.9 indicates highest confidence

(*OsGA2ox3* and *GA3ox2*) and signaling associated genes (D1, GIDI and SLR1).

Our results revealed that mutation resulted in the deletion of large fragments between the two targets could lead to a significant decrease in PH and endogenous GA level (Table 2). The mutant plants were highly responsive to exogenous GA_3 and wild phenotype with normal PH was observed in all mutants with the treatment of exogenous GA_3 (Fig. 7). These results showed that normal height of rice plant can be restored by application of exogenous GA_3 treatment and thus GA signaling pathways are main targets for further improvement of crop yield (Hedden 2003; Qiao and Zhao 2011). Each of the mutants showed different levels of endogenous GA which was strongly correlated with *SD1*

expression level. The results showed that the CRISPR/Cas9-mediated double-target mutation in the first exon of *SD1* and modulation of GA biosynthesis is important to obtain desirable plant architecture to address food security issues (Spielmeyer et al. 2002a, b). *SD1* mutations also resulted in distributed cell layers and prevention of second internode elongation. It was found from the paraffin sections of the second internodal cells that the mutant showed significant decrease in cell length, width and size, while increased cell numbers as compared with the WT cells. The decrease in length, width and size of longitudinal cells may be the major cause reduced PH. Dwarf mutant plants generally showed affected cell expansion, which is related to the development of cell number and size (Li et al. 2011).

The stem protein of WT and mutant line (G7-6-1) were separated by 2-DE gel electrophoresis and 30 differential spots were exercised and total 24 proteins were identified. The identified proteins were involved growth regulation, protein biosynthesis, photosynthesis, environmental and nutrient stress responses, photorespiration and metabolisms of carbon, sulfur and energy pathways.

Cytosolic glyceraldehyde-3-phosphate dehydrogenase (GAPDH), glutamate dehydrogenase, ATP synthase subunit alpha 1, putative glyceraldehyde 3-phosphate de-hydrogenase, putative caffeoyl-coA *O*-methyltransferase, putative isoflavone reductase, probable photosystem II oxygen-evolving complex protein 2 precursor, aminotransferase-like and Lactoylglutathione lyase are involved in photosynthesis and confers abiotic stress tolerance in rice and other plants (Zhang et al. 2005; Qiu et al. 2009; Kim et al. 2010; Zhang et al. 2011). Aldehyde dehydrogenase (ALDHs) catalyze the irreversible oxidation of a wide range of reactive aldehydes to their corresponding carboxylic acids and plays a role in detoxifying the aldehydes generated by environmental stresses (Kirch et al. 2004, 2005; Mustafiz et al. 2011). Abscisic stress-ripening protein 5 involved in GA signaling and regulation of plant growth in the basal region of leaf sheaths. It also regulates the expression of different genes that collectively contributes to the protection of the cell in aluminum stress (Takasaki et al. 2008; Arenhart et al. 2013). Actins are highly conserved proteins that are involved in cytoplasmic streaming, cell shape determination, cell division, organelle movement and extension growth and are ubiquitously expressed in all eukaryotic cells. Fructose-bisphosphate aldolase 1, cytoplasmic-fructose-bisphosphate aldolase plays a key role in glycolysis and gluconeogenesis and involved in GA-mediated root growth (Konishi et al. 2004). UDP-glucuronic acid decarboxylase enzyme plays a significant role in rice seed development at a certain stage (Suzuki et al. 2004). Gibberellin 20 oxidase 1 (*GA20OX1*) is a key oxidase enzyme in the biosynthesis of gibberellin. Catalyzes the conversion of GA12 and GA53 to GA9 and GA20, respectively, via a three-step oxidation at C-20 of the GA skeleton. Plants over-expressing *GA20OX1* have high levels of bioactive GA and exhibit an overgrowth phenotype with increased internode elongation and leaf sheath length (Toyomasu et al. 1997; Oikawa et al. 2004). Gibberellin 20 oxidase 2 is key oxidase enzyme in the biosynthesis of GA that catalyzes the conversion of GA53 to GA20 via a three-step oxidation at C-20 of the GA skeleton and contributes to the promotion of internode elongation in response to submergence via ethylene and GA signaling (Spielmeyer et al. 2002a, b). Gibberellin 2-beta-dioxygenase 3 converts GA1, GA20, and GA29 to the corresponding 2-beta-hydroxylated products GA8, GA29-catabolite, respectively, and catalyzes the 2-beta-hydroxylation of several biologically active GAs, leading to the homeostatic regulation of their endogenous level. Catabolism of GAs plays a central role in plant development (Sakai et al. 2003; Lo et al. 2008). In summary, among 24 proteins, there were five proteins such as Glyceraldehyde-3-phosphate dehydrogenase, Putative ATP synthase, Fructose-bisphosphate aldolase 1, *S*-adenosyl methionine synthetase 1 and Gibberellin 20 oxidase 2 were downregulated in mutant line which play an important role in plant growth by controlling the GA level, photosynthesis and energy, and absence or downregulation of these proteins may be the cause of reduced PH.

The natural mutants, *sd1-c, sd1-j, sd1-r and sd1-EQ* are caused by weak alleles resulting from substitutions in exon regions along with *sd1-d* exhibit semi-dwarfism with normal setting. The deletion in *sd1-d* might form a specific weak allele rather than reflect the gene function. Gene function was mainly studied in previous research, such as in the C20-GA2ox family (Monna et al. 2002; Sasaki et al. 2002; Spielmeyer et al. 2002a, b; Asano et al. 2011). It is still unclear, whether these phenotypic differences are due to the different genetic backgrounds or experimental approaches. In another respect, some severe defects caused by mutation may have already been eliminated and the phenotypes we observed in the corresponding mutants nowadays may have been the outcome of selection during artificial breeding. These differences suggest that research on the true functions of genes can be further improved with more mutation types, phenotypes and genetic backgrounds being taken into consideration. In our study all the resulted loss-of-function mutants exhibited almost same phenotype.

In conclusion, reduction in PH by modifying the *SD1* expression in rice plants to affect the GA biosynthesis is potentially of high agronomic interest. Dwarfing controlled by different mechanisms as presented in this study and previous reports showed loss of function *SD1* leads to dwarfism with altered endogenous GA levels and is a great source for future breeding program to broad the genetic base of PH. This study showed the genetic mutations are not only helpful to improve the plant characters, it also helps to understand the mechanism underlying the

biochemical behavior changes in cell of the plants. The semi-dwarf mutant lines successfully obtained in this study can be exploited for future breeding programs.

Conclusions

In summary, CRISPR/Cas9-mediated mutation in the *SD1* allele in the elite cultivated *Indica* rice successfully developed new rice lines with modified plant architecture having short PH and slightly improved grain yield, while maintaining all the quality characters. The CRISPR/Cas9 technology induces fastest changes to plant genome than other molecular approaches and mutations passed to the next generations without any rewriting or emendations. The resulted loss-of-function *SD1* phenotype suggests that bioactive GA production for shoot elongation is severely defective, but that for reproductive development is less affected. The dwarf mutants facilitated the discovery of molecular mechanism underlying the GA biosynthesis and signaling. All the data in this study provide an example of gene editing directly in elite crop varieties with potential for commercialization. This study will help to expand unexplored angles of rich natural biological diversity and further study is needed to discover the experimental avenues for transcriptome analysis to improve the experimental application of CRISPR/Cas9 in future research.

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Author contributions HY, KT and GN conceived, designed and performed the experiments. FX was responsible for vector construction and LL contributed to field preparations. WX, FX, GN and BU recorded the data and tested guide sequences. GN and BU responsible for data analysis and wrote the paper. LY provided vector and supervised the transformation and supervised the methodology. LR visualized the project, supervised the research, funding acquisition, feedback on data presentation and reviewed the final draft. All the authors have read the manuscript and approved the submission.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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