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Upgrading the genome of an elite japonica rice variety Kongyu 131 for lodging resistance improvement

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Summary

Developing a new rice variety requires tremendous efforts and years of input. To improve the defect traits of the excellent varieties becomes more cost and time efficient than breeding a completely new variety. Kongyu 131 is a high-performing *japonica* variety with early maturity, high yield, wide adaptability and cold resistance, but the poor-lodging resistance hinders the industrial production of Kongyu 131 in the Northeastern China. In this study, we attempted to improve the lodging resistance of Kongyu 131 from perspectives of both gene and trait. On the one hand, by QTL analysis and fine mapping we discovered the candidate gene loci. The following CRISPR/Cas9 and transgenic complementation study confirmed that *Sd1* dominated the lodging resistance and favourable allele was mined for precise introduction and improvement. On the other hand, the *Sd1* allelic variant was identified in Kongyu 131 by sequence alignment, then introduced another excellent allelic variation by backcrossing. Then, the two new resulting Kongyu 131 went through the field evaluation under different environments, planting densities and nitrogen fertilizer conditions. The results showed that the plant height of upgraded Kongyu 131 was 17%–26% lower than Kongyu 131 without penalty in yield. This study demonstrated a precise and targeted way to update the rice genome and upgrade the elite rice varieties by improving only a few gene defects from the perspective of breeding.

Introduction

Rice is the staple food for more than 60% Chinese population and nearly 40% of the nation's calorie intake is contributed by rice (Zeng *et al.*, 2017). Kongyu 131 is an elite *japonica* rice variety of northeast China with excellent characteristics, such as early maturity, high yield, wide adaptability and cold resistance. It has been popularized in northeast China since 1997, and has gradually withdrawn from production in the last decade. Now it is only grown in small paddies. Its cumulative planting area exceeds 7 million hectares. With the frequent reoccurring extreme weather, such as typhoons and storms, the problem of rice lodging at the late maturity stage has brought attention to the rice breeders (Ishimaru *et al.*, 2008; Nomura *et al.*, 2019). In rice breeding, reducing plant height has been one of the main targets to improve lodging resistance. The rice Green Revolution has led to an unprecedented increase in rice production since 1960s, which saves the world from imminent famine. The introgression of *semi-dwarf 1* (*sd1*) have been proved functional in preventing or mitigating rice lodging even under a significant amount of nitrogen fertilization (Khush, 2001). The *sd1* gene has been extensively used for breeding *indica* cultivars, and multiple *sd1* alleles have been reported (Bhuvaneswari *et al.*, 2020; Sha *et al.*, 2022; Zhang *et al.*, 2020). The use of *sd1* alleles and corresponding germplasm resource remain unclarified for breeding *japonica* rice.

Lodging is one of the major negative traits that impairs rice yield, quality and mechanical harvest (Lang *et al.*, 2012; Setter *et al.*, 1997). A number of genes and quantitative trait loci associated with lodging resistance have been identified and characterized (Chen *et al.*, 2018; Ishimaru *et al.*, 2008; Okuno *et al.*, 2014; Ookawa *et al.*, 2010, 2016; Wang and Wang, 2017). The recessive *sd1–d* gene conferring the semi-dwarf phenotype was firstly identified from the Chinese cultivar Dee-Geo-Woo-Gen (DGWG), which was the causal gene driving the rice Green Revolution (Monna *et al.*, 2002; Sasaki *et al.*, 2002). *SD1* encodes the enzyme, GA20-oxidase 2, which can convert GA12/GA53 to the bioactive GA precursors GA9/GA20 in the gibberellin (GA) biosynthesis pathway. The *sd1–d* allele carries a 383-bp deletion across exon 1 to exon 2 which leads to a truncated polypeptide. The *sd1–d* causes defective GA20-oxidase activity, thus resulting in shortened culm trait (Sasaki *et al.*, 2002; Spielmeyer *et al.*, 2002).

Although numerous genes and QTLs have been identified with strong association with plant height, independent global semi-dwarf rice breeding practices still utilize *SD1* alleles. It is mainly because *SD1* affects only plant height while the other dwarf traits are often accompanied by other undesired adverse effects, such as reduced fertility and lower yield (Hirano *et al.*, 2017). To date, seven *sd1* haplotypes are found in *indica* rice and four in *japonica* rice (Sha *et al.*, 2022). Considering the *SD1* of Kasalath (*indica cv.*) as wild type, the *sd1-AJNT* allele from Ai-Jiao-Nan-Te contains a GC deletion in exon 1, and the frameshift mutation leads to a premature stop codon in the downstream (Sasaki *et al.*, 2002; Spielmeyer *et al.*, 2002). The *sd1-bm* allele from Pusa 1652 has a single base substitution in exon 3 also causing a premature stop codon (Bhuvaneswari *et al.*, 2020). *Sd1–d*, *sd1–AJNT* and *sd1-bm* are all regarded as gene knockout alleles,

and in particular *sd1–d* has been widely used in the *indica* rice breeding program in south and southeast Asian regions (Asano *et al*., 2007). The *SD1–EQ* in Nipponbare (*japonica* cv.) harbours two single nucleotide polymorphisms (SNPs) in exon 1 and exon 3 (E100G and Q340R), resulting in lower enzyme activity (Asano *et al*., 2011). *SD1–EQ* has been preserved in most *japonica* rice cultivars after artificial Selection during the long-term domestication (Yu *et al*., 2020).

The *sd1–d* allele has been introduced in the *japonica* rice breeding to produce Kinuhikari and Dontokoi in Japan (Asano *et al*., 2007). However, whether this loss of function allele type can be used in *japonica* rice breeding in northeast China is not clear. With the increasing demand of lodging resistance for rice industry, the *sd1* alleles have brought attention to *the japonica* rice breeding program. The advanced backcross QTL (AB–QTL) analysis has been adopted mining the associated genes for desired traits in multiple germplasms (Alyr *et al*., 2020; Kunert *et al*., 2007; Sayed *et al*., 2012; Talukder *et al*., 2020). In recent years, with the rapid development of genome editing technology, CRISPR/Cas9 has been used to generate semi-dwarf lines in some elite landraces (Biswas *et al*., 2020; Hu *et al*., 2019).

In this study, we aimed to improve the lodging resistance of Kongyu 131 by updating the genome of Kongyu 131. The upgraded variety of Kongyu 131 exhibited lower plant height with normal yield under high-nitrogen fertilization rate. The improvement of lodging resistance can potentially reduce the labor cost of rice cultivation by adopting simple cultivation methods, such as direct seeding. This study provided an insight into the adoption of advanced breeding technology to improve the adaptation of existing rice varieties for future agricultural production.

Results

AB-QTL analysis for plant height

The plant height was analysed using the BC_3F_2-038 population (GKAR × Kongyu 131, including 86 individuals) in 2015 (Figure 1a). The BC_3F_2–038 population was derived from BC_3F_1-08A01 with 129 SNP markers on 12 chromosomes. The AB–QTL analysis indicated BC_3F_1-08A01 carried four fragments from GKAR on chromosome 1, 5, 8 and 9, respectively (Figure 1b). The AB–QTL analysis indicated the QTL associated with plant height was at the interval of SNP35005–SNP40573, near 35–40 Mb on chromosome 1 (Figure 1c). This QTL (namely *qKD1*) can explain 55.7% of phenotypic variation of plant height. The *qKD1* from GKAR contributed to the reduced plant height with additive effect of −9.51 and LOD of 15.19. The plant height analysis also showed that heterozygote (+/–) and homozygote (+/+) of *qKD1*–GKAR were significantly lower than that of Kongyu 131 and homozygote of *qKD1*–Kongyu 131 (–/–) with partial dominance observed (Figure 1d).

Fine mapping of *qKD1*

The heterozygous line, BC_3F_3-622F08, at *qKD1* locus showed 97.1% recovery ratio of the recurrent parent genome (RRPG) (Figure 2a). The QTL analysis on 43 selected recombinant lines at *qKD1* locus derived from BC_3F_3-622F08 indicated the target QTL which was within the 917 kb region between SNP39656 and SNP40573 (Figure 2b). A heterozygous *qKD1* line was selected to produce 1344 self-crossed progenies. With extra 33 SNP markers added to this QTL interval, 19 recombinant lines were selected for the progeny test, and the target QTL was further narrowed down to the 11.9 kb region between SNP40137 and SNP40149 (Figure 2c). In this region, the two annotated genes were Os01g0883800 and Os01g0883900 (Figure 2d). Os01g0883800 was known as the *SD1* gene.

Sequence alignment of different *SD1* alleles

The *SD1* sequence alignment of Nipponbare, Kongyu 131, GKAR and IR64 was shown in Figure 3. The coding sequence (CDS), 5′-UTR and 3′-UTR were identical between Kongyu 131 and Nipponbare. While *sd1*–GKAR carried three SNPs, including two non-synonymous SNPs (Glu100Gly and Gln340Arg) and a C to G mutation in the 3rd exon resulting a premature stop codon, which was considered as a loss function mutation. The gene sequence of *sd1* from IR64 was the same as the previously reported *sd1–d* allele.

The plant height of Kongyu 131 *SD1*-knockout mutants reduced significantly

To develop the *SD1*-knockout mutants of Kongyu 131, target1 and target2 were selected to design gRNA1 and gRNA2 near the C to G mutation in the 3rd exon. A total of 27 positive transgenic lines were obtained and the DNA sequencing results showed that all 27 T_0 plants contained mutation at the target sites, and 4 were homozygous designated as T_0-1, T_0-2, T_0-3 and T_0-4, respectively (Figure 4a). T_0-1 had 1-nt insertion (T) at both the target1 and target2. T_0-2 had 1-nt insertion (A) at target1 and 1-nt deletion (T) at target2. T_0-3 had 1-nt insertion (C) in target1 and 1-nt insertion (A) at target2. T_0-4 only had 1-nt insertion (T) at target1. The self-pollinated progenies (T_1-1, T_1-2, T_1-3 and T_1-4) were obtained to evaluated the plant height. All the *SD1*-knockout mutants showed a remarkable ~20 cm reduction in the plant height compared with Kongyu 131 (Figure 4b).

Complementary *SD1* gene could rescue the plant height of the introgression line

In order to further verify that the plant height gene at *qKD1* locus is *SD1*, the complementary experiment of *SD1* gene and another gene Os01g0883900 located in *qKD1* locus was carried out in this study. The full-length sequences of *SD1* (A 6147-bp fragment including a 2017-bp sequence upstream of the start code and a 1387-bp sequence downstream of the stop code) and Os01g0883900 (A 7769-bp fragment including a 2147-bp sequence upstream of the start code and a 1302-bp sequence downstream of the stop code) in Kongyu 131 were amplified to construct complementary vectors, respectively. The resulting *SD1* and Os01g0883900 complementary vectors were transformed into a *qKD1*–GKAR near-isogenic line (NIL) in Kongyu 131 background. The results showed that plant height of the *SD1* complementary line was increased, while the *Os01g0883900* complementary line remained the same with NIL (Figure 4c). Combined with the results of CRISPR/Cas9 gene editing, it was proved that the plant height gene in *qKD1* locus was *sd1*.

Developing single segment substitution line carrying *sd1–GKAR* and *sd1–d* allele

In order to evaluate the effect of *sd1* allele from GKAR on plant height of Kongyu 131, BC_3F_3-621A02 was selected from the BC_3F_3 population according to the genotype of marker M1–M5 designed near *sd1* locus. The recombination occurred between markers M1 & M2, M4 & M5, and the length of introduced fragment was approximate 665 kb. Simultaneously, a

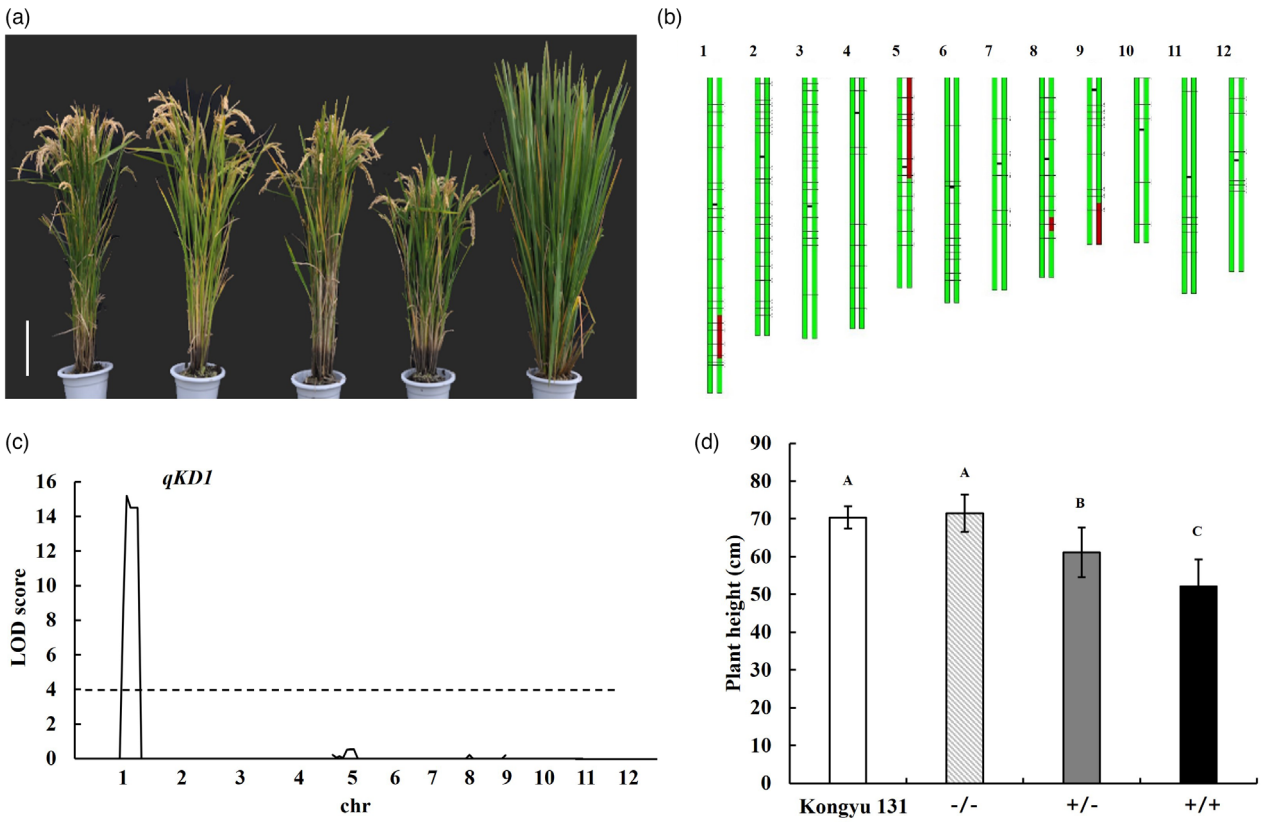

Figure 1 QTL analysis of the subpopulation BC_3F_2-038 derived from BC_3F_1-08A01 with Kongyu 131 as the recipient parent and GKAR as the donor. (a) Different individuals in mapping population, Kongyu 131 (left), GKAR (right), Scale bar denotes 20 cm; (b) Graphical genotype of BC_3F_1-08A01. The green and red bars represent the chromosome segments derived from Kongyu 131 and GKAR, respectively; The horizontal black lines indicate the SNP markers; (c) QTL analysis for plant height in BC_3F_2-038; (d) Plant height comparison of individuals with different genotype at *qKD1* locus in BC_3F_2-038 population. –/–, +/– and +/+ represents homozygote for Kongyu 131, heterozygote and homozygote for GKAR. The capital letters (a, b and c) represent significant difference at $p < 0.01$ based on ANOVA.

homozygous fragment and a heterozygous fragment from GKAR were detected on chromosome 4 and 12, respectively, with a RRPG of approximate 96.41% (Figure 5a). BC_4F_1-308D03, with the target fragment, selected from 43 backcross progenies of BC_3F_3-621A02 also carried a heterozygous fragment from GKAR on chromosome 4, eliminating the non-target fragment on chromosome 12 (Figure 5b). Eventually, BC_4F_2-20B02 was selected from 64 self-crossed progenies of BC_4F_1-308D03 with no non-target fragment contamination (Figure 5c). To further shorten the target fragment length, the markers used for *qKD1* fine mapping were added to screen recombinants. BC_4F_4-259B04 was selected and named SSSL–*sd1*–*GKAR*, which carried a homozygous fragment from GKAR at the *sd1* locus (Figure 5d). The fragment was approximately 384 kb and the RRPG was about 99.96% based on the analysis of 252 markers on 12 chromosomes.

SSSL BC_5F_1-22L01 carrying *sd1*–*d* from IR64 was selected from IR64 × Kongyu 131 population of 186 lines. According to the 210 SNP markers on 12 chromosomes, BC_5F_1-22L01 only carried one target fragment with length of about 14 Mb and RRPG of 98.49%. To shorten the target fragment length, 5 markers M1–M5 designed in the vicinity of *sd1* were used to screen the recombinants. From the successive selfing progenies of BC_5F_1-22L01, BC_5F_3-1057G04 (Figure S1a), BC_5F_4-268A03 (Figure S1b), BC_5F_5-33A05 (Figure S1c) and BC_5F_6-168A01 (Figure S1d) were selected at each generation. BC_5F_6-168A01 was confirmed carrying a homozygous fragment from IR64 at the *sd1* locus. The was approximate 917 kb and the RRPG was about 99.94%. BC_5F_6-168A01 was named SSSL–*sd1*–*d*.

Comparison of agronomic characters of two SSSLs under different locations

In order to evaluate the field performance of SSSLs with two different *sd1* alleles, we conducted 3 years of field experiments with four cultivation densities and three fertility levels at the Jiamusi experimental base. Under A1 density, PH of the two SSSLs showed a significant reduction compared with Kongyu 131 for three consecutive years (Table 1). The PH of Kongyu 131 was the highest (72.9 cm), followed by SSSL–*sd1*–*GKAR* (57.4 cm) and SSSL–*sd1*–*d* (47.5 cm). According to the LI analysis, SSSL–*sd1*–*d* was the most resistant to lodging and SSSL–*sd1*–*GKAR* was moderate compared with Kongyu 131 (Table 1).

The YP of Kongyu 131 and SSSL–*sd1*–*GKAR* was higher than SSSL–*sd1*–*d* in particular for the 2018 and 2019 crop years. No significant difference was found in GNP and HGW between Kongyu 131 and SSSL–*sd1*–*GKAR*, while GNP and HGW were significantly lower for SSSL–*sd1*–*d*. No significant and consistent difference was found in ETP and SSR comparing Kongyu 131, SSSL–*sd1*–*GKAR* and

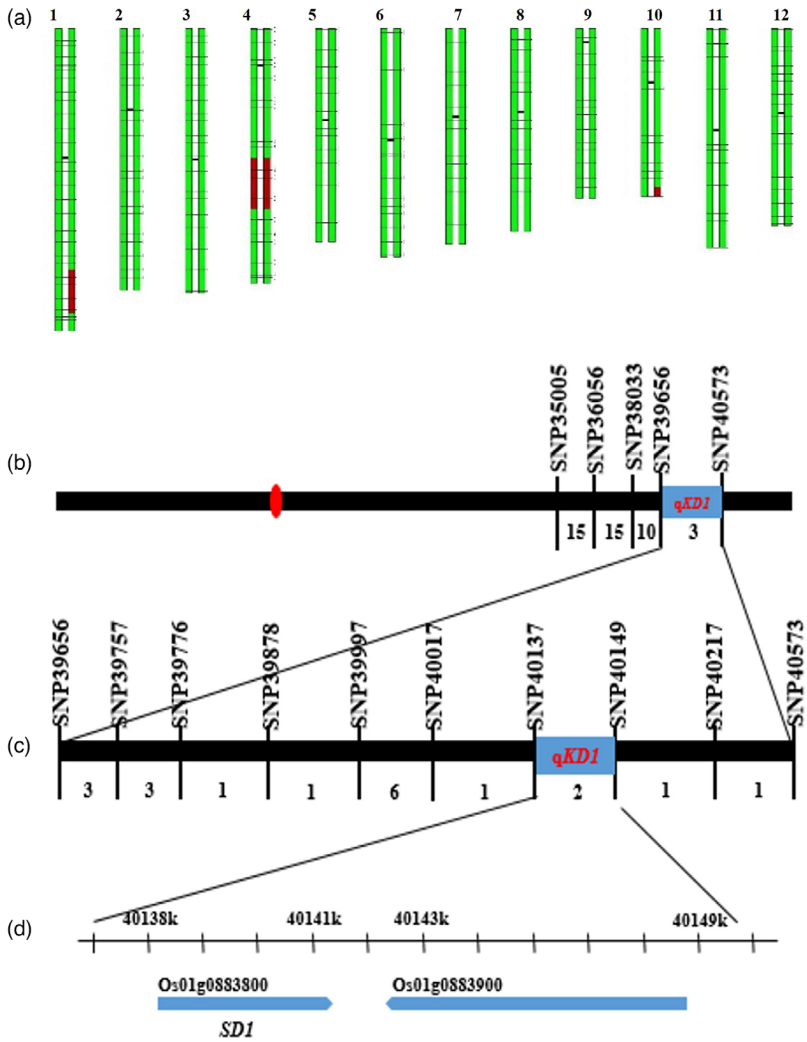

Figure 2 Fine-mapping of plant height gene within *qKD1*. (a) Graphical genotype of BC_3F_3-622F08; (b) The *qKD1* was narrowed between SNP39656 and SNP40573; (c) The *qKD1* was further narrowed down between SNP40137 and SNP40149; (d) Two annotated genes *Os01g0883800 (SD1)* and *Os01g0883900* were found in the 11.9 kb region between SNP40137 and SNP40149.

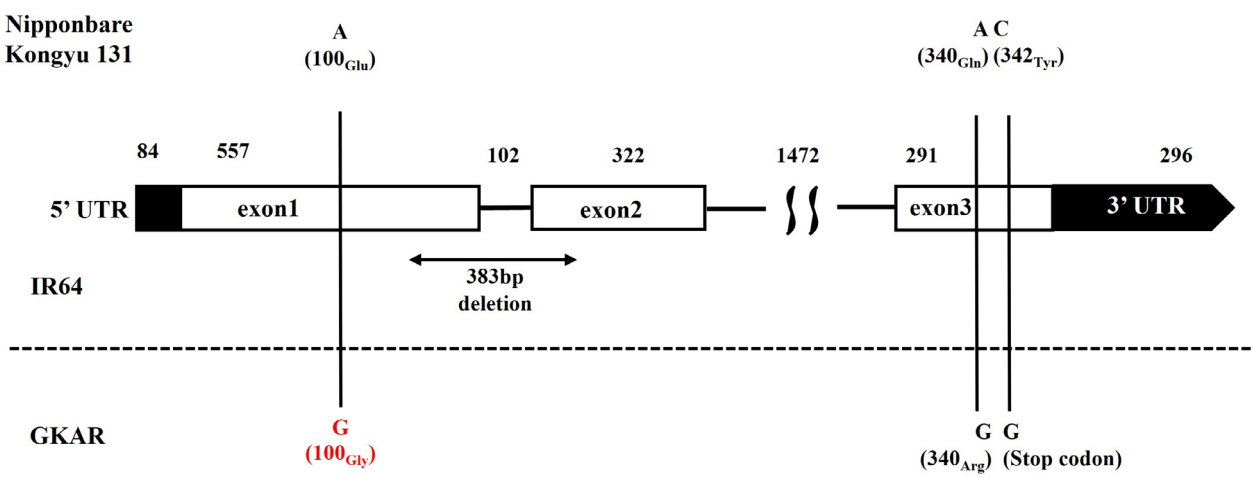

Figure 3 *SD1* structure and allelic variation in Kongyu 131, Nipponbare, IR64 and GKAR. Black vertical lines denote single nucleotide substitutions leading to amino acid changes (in parentheses). The horizontal arrow denotes the sequence deleted in *sd1–d*. The numbers above show length of the 5′ UTR, exons, introns and 3′ UTR.

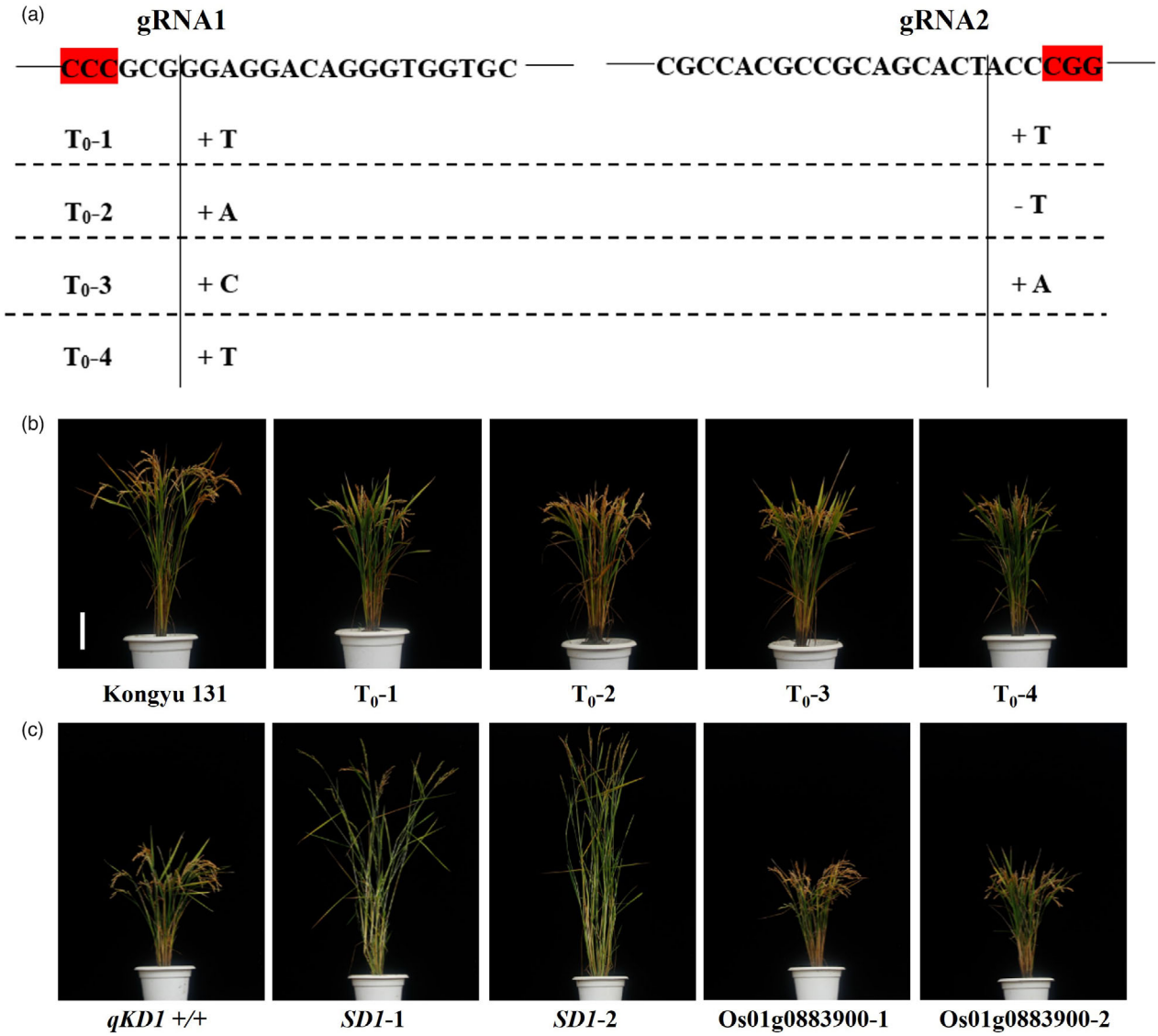

Figure 4 Sequence variations of mutants obtained by CRISPR/Cas9 and plant height performance of Kongyu 131, mutants and transgenic complementary lines. (a) Nucleotide sequence change of four homozygous T_0 mutant near two target sites. The red boxes represent PAM; (b) Plant height comparison of Kongyu 131 and four T_0 mutants obtained by CRISPR/Cas9. (c) Plant height comparison of near-isogenic line (NIL) with homozygous GKAR segment at *qKD1* locus, and transgenic complementary individuals transferring *SD1* and *Os01g0883900* gene into NIL. Scale bar = 20 cm.

SSSL–*sd1–d* (Table 1). The yield difference among the three lines can be attributed to the difference in GNP and HGW.

Comparison of agronomic characters of two SSSLs under different cultivation densities

Comparing the different cultivation densities in field experiments in 2019 and 2020, it showed Kongyu 131, SSSL–*sd1–GKAR* and SSSL–*sd1–d* were significantly different in PH and LI (Table 2). The PH and LI of Kongyu131 was the highest, followed by SSSL–*sd1–GKAR* and SSSL–*sd1–d* regardless of the cultivation density. LI increased with the density increased, indicating that the lodging resistance was negatively correlated with planting density. This suggested the cultivars with strong lodging resistance were more suitable for compact planting.

Under the same cultivation density, YP of Kongyu 131 and SSSL–*sd1–GKAR* were the same but significantly higher than SSSL–*sd1–d*. In terms of yield components, Kongyu 131 and two SSSLs had similar ETP and SSR, but the GNP of Kongyu 131 and SSSL–*sd1–GKAR* was significantly higher than SSSL–*sd1–d*. These results showed that the introgression of *sd1–d* allele decreased GNP and YP. With the increase of cultivation density, ETP and HGW of all three cultivars decreased significantly, which resulted in the reduced YP (Table 2, Figure S2). Under different cultivation densities, the lodging resistance of SSSL–*sd1–GKAR* was higher than that of Kongyu 131, and the YP was the same with Kongyu 131. This suggested SSSL–*sd1–GKAR* with enhanced lodging resistance was potentially an improvement of Kongyu 131 when the cultivation density was higher.

Comparison of agronomic characters of two SSSLs under different levels of nitrogen supply

The yield related crop performance of Kongyu 131 and the two SSSLs under three nitrogen levels at B3 planting density in 2019, were shown in Table 3 and Figure S3. Kongyu 131 had the

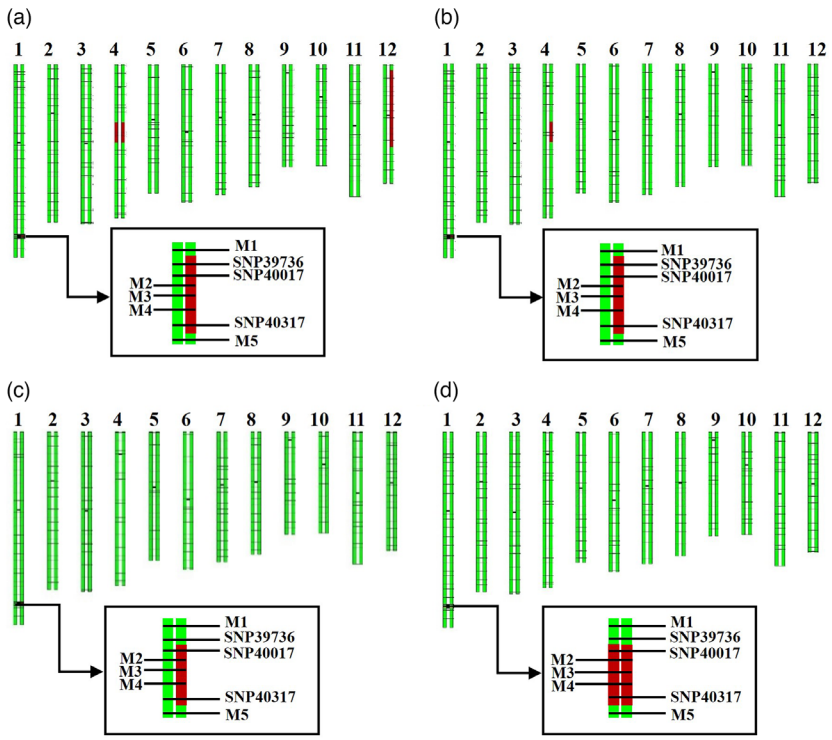

Figure 5 Graphical genotype of the selected lines. (a) BC_3F_3-621A02; (b) BC_4F_1-308D03; (c) BC_4F_2-20B02; (d) BC_4F_4-259B04. The green and red bars denote the chromosome fragments derived from Kongyu 131 and GKAR, respectively. The horizontal black lines denote the SNP markers (the shorter, darker lines represent the centromeres).

highest PH and the largest LI, while SSSL–*sd1–d* had the lowest PH and the highest lodging resistance. The PH and LI of SSSL–*sd1–GKAR* were moderate compared with Kongyu 131 and SSSL–*sd1–d*. The ETP of the three cultivars increased with the nitrogen level increased, with the highest observed in SSSL–*sd1–d*. Kongyu 131 and SSSL–*sd1–GKAR* were comparable in YP and yield components but significantly higher in ETP, HGW and YP than SSSL–*sd1–d*, suggesting that SSSL–*sd1–GKAR* had superior lodging resistance than Kongyu 131 under high-nitrogen application.

Discussion

Comparing with developing a new rice variety, to improve the defect traits of the existing industrial varieties becomes more cost-effective with the advantage of emerging new breeding technologies. Our team has devoted to improve the undesired traits of Kongyu 131 by targeted introgression of gene alleles of superior traits, such as rice blast resistance, spikelet number per panicle, grain size and heading date (Feng *et al*., 2017, 2019; Nan *et al*., 2018; Wang *et al*., 2019). In the current study, we focus on solving the lodging issue of Kongyu 131 at the late maturity stage. The demand of varieties with high-lodging resistance continues to rise to support the direct-seeded planting and other high-yield cultivation strategies.

In *indica* cultivars, semi-dwarf gene *sd1* has been widely selected in the breeding program to improve the lodging resistance. While in *japonica* rice, the corresponding dwarf genes can be divided into two groups. One is single major gene which is often non-allelic in different *japonica* rice varieties, and accompanied by poor agronomic characters, such as severe dwarfism, short panicle length,and small or sterile grain (Ferrero-Serrano *et al*., 2019). The poor-recombination ability between dwarfing and other traits of the parents makes the dwarfing genes difficult to be used in *japonica* rice breeding (Rana *et al*., 2021; Souza and Tavares, 2021). The other is multiple minor genes which can be lost easily in the process of progeny selection and requires multiple generations of re-selection to fix the minor genes. The interaction between multiple minor genes and other agronomic trait also needs to be considered in the selection (Babu *et al*., 2020). Different *SD1* alleles have been adopted to achieve different culm heights in *indica* rice dwarf-breeding. However, almost all *japonica* rice contains weak functional allele *SD1–EQ*, and the nucleotide diversity at the *SD1* locus in *japonica* rice decreased dramatically, which was not found in the *indica* landrace or wild ancestor (Asano *et al*., 2011). This indicates that the *SD1* locus has been subjected to artificial selection in the early domestication and preserved in *japonica* rice. Therefore, the current study attempted to improve the lodging resistance of Kongyu 131 by targeted major gene introgression of *indica* type *sd1* allele.

In this study, the novel allele *sd1–GKAR* identified in GKAR was found different from the other previously reported *indica* alleles, which required evaluation for rice breeding (Bhuvaneswari *et al*., 2020). The NILs containing different *sd1–GKAR* and *sd1–d* were developed to evaluate the allelic effect on lodging resistance and selected yield related agronomic traits in the Kongyu 131 background Although *sd1–d* allele was widely used in *indica* rice breeding, the introduction into Kongyu 131 led to a significant decrease in yield in Kongyu 131 background. While the introduction of *sd1–GKAR* resulted in a moderate reduction in plant height with no yield penalty in Kongyu 31 background. These variation in yield can possibly explained by the variation in the mutagenesis. The 383-bp deletion from exon 1 to exon 2 in *sd1–d* eliminated the GA20ox-2 activity in the NIL. In contrast, with the 3 SNPs in the coding region, *sd1–GKAR* encoded a truncated GA20ox-2 variant with two amino acid substitutions which led to the reduced enzyme activity. Another possibility is

Table 1 Agronomic performance of Kongyu 131, SSSL–*sd1*–*GKAR* and SSSL–*sd1*–*d* in 2018–2020

	2018			2019			2020		
Traits	Kongyu 131	SSSL–*sd1*–*GKAR*	SSSL–*sd1*–*d*	Kongyu 131	SSSL–*sd1*–*GKAR*	SSSL–*sd1*–*d*	Kongyu 131	SSSL–*sd1*–*GKAR*	SSSL–*sd1*–*d*
PH (cm)	75.7 ± 2.9^{A}	55.9 ± 2.3^{B}	48.6 ± 3.4^{C}	75.5 ± 2.0^{A}	60.6 ± 2.1^{B}	48.6 ± 1.5^{C}	67.5 ± 1.9^{A}	55.8 ± 1.5^{B}	45.4 ± 2.5^{C}
LI	5.77 ± 1.19^{Aa}	3.72 ± 1.18^{Bb}	2.62 ± 0.54^{Bc}	6.05 ± 1.83^{Aa}	4.09 ± 1.05^{ABb}	3.63 ± 1.37^{Bb}	2.33 ± 0.35^{Aa}	1.89 ± 0.26^{Ab}	1.05 ± 0.20^{Bc}
ETP	34.8 ± 5.6	34.3 ± 4.6	35.2 ± 4.0	31.2 ± 2.8	37.5 ± 12.0	37.9 ± 2.8	22.8 ± 4.6^{B}	20.4 ± 1.7^{B}	31.2 ± 2.0^{A}
GNP	122.1 ± 22.6^{ABa}	124.3 ± 24.7^{Aa}	98.2 ± 16.8^{Bb}	120.5 ± 29.5^{A}	128.7 ± 17.6^{A}	92.0 ± 13.7^{B}	105.8 ± 15.5^{A}	104.6 ± 10.5^{A}	73.4 ± 12.0^{B}
SSR (%)	96.5 ± 3.6	95.4 ± 3.0	95.5 ± 2.6	92.6 ± 6.6	93.5 ± 3.5	92.7 ± 8.9	96.8 ± 2.8^{A}	95.8 ± 0.8^{A}	88.4 ± 4.2^{B}
HGW (g)	2.40 ± 0.10^{A}	2.37 ± 0.08^{A}	2.23 ± 0.07^{B}	2.46 ± 0.10	2.40 ± 0.08	2.39 ± 0.08	$2.62^{a} \pm 0.12$	$2.58^{ab} \pm 0.10$	$2.45^{b} \pm 0.07$
YP (g)	61.00 ± 9.68^{A}	55.05 ± 6.07^{A}	42.20 ± 4.12^{B}	59.34 ± 7.45^{A}	59.95 ± 11.70^{A}	40.24 ± 4.58^{B}	46.91 ± 7.09	43.21 ± 4.94	39.23 ± 4.37

ETP, effective tillers per plant; GNP, grain number per panicle; HGW, hundred grain weight; LI, lodging index; PH, plant height; SSR, seed setting rate; YP, yield per plant. Data presented as the means with standard deviations were obtained from 12 plants. The letter A/B/C, a/b/c represent significant difference at $P < 0.01$ and $P < 0.05$, respectively, based on ANOVA.

Table 2 Agronomic performance of Kongyu 131, SSSL–*sd1*–*GKAR* and SSSL–*sd1*–*d* under different cultivation densities in 2019–2020

	A3			B3			C3		
Traits	Kongyu 131	SSSL–*sd1*–*GKAR*	SSSL–*sd1*–*d*	Kongyu 131	SSSL–*sd1*–*GKAR*	SSSL–*sd1*–*d*	Kongyu 131	SSSL–*sd1*–*GKAR*	SSSL–*sd1*–*d*
PH (cm)	76.2 ± 4.1^{A}	60.1 ± 3.4^{B}	49.3 ± 2.3^{C}	72.7 ± 6.7^{A}	59.3 ± 2.8^{B}	47.2 ± 2.9^{C}	80.5 ± 6.5^{A}	57.9 ± 3.7^{B}	49.4 ± 2.2^{C}
LI	3.99 ± 1.27^{Aa}	3.11 ± 0.90^{Ab}	1.98 ± 0.71^{Bc}	4.37 ± 1.60^{A}	3.52 ± 1.20^{A}	2.12 ± 0.68^{B}	4.87 ± 2.34^{Aa}	3.14 ± 1.74^{ABb}	2.33 ± 1.09^{Bb}
ETP	33.3 ± 6.3	34.5 ± 6.1	34.3 ± 11.5	22.2 ± 7.1	21.2 ± 4.6	22.1 ± 4.2	14.5 ± 3.4	15.4 ± 4.0	14.1 ± 2.6
GNP	103.5 ± 20.4^{A}	110.1 ± 23.5^{A}	80.9 ± 18.3^{B}	102.2 ± 15.4^{A}	106.6 ± 31.1^{A}	70.6 ± 14.4^{B}	99.9 ± 21.2^{A}	96.9 ± 21.3^{A}	62.7 ± 17.2^{B}
SSR (%)	92.6 ± 4.8	95.5 ± 2.7	93.3 ± 5.7	95.0 ± 3.5	93.9 ± 6.4	90.4 ± 7.2	94.8 ± 3.7	93.6 ± 2.7	90.6 ± 10.4
HGW (g)	2.48 ± 0.15	2.44 ± 0.13	2.47 ± 0.11	2.41 ± 0.12^{A}	2.39 ± 0.07^{A}	2.27 ± 0.09^{B}	2.26 ± 0.20^{a}	2.35 ± 0.11^{a}	2.23 ± 0.10^{b}
YP (g)	52.84 ± 8.14^{A}	54.80 ± 9.51^{A}	40.29 ± 11.36^{B}	33.57 ± 6.94^{A}	30.95 ± 8.21^{A}	22.07 ± 5.70^{B}	19.75 ± 3.47^{A}	21.56 ± 6.96^{A}	14.07 ± 3.54^{B}

ETP, effective tillers per plant; GNP, grain number per panicle; HGW, hundred grain weight; LI, lodging index; PH, plant height; SSR, seed setting rate; YP, yield per plant. A3, B3 and C3 denote different cultivation densities. A3: each plot consisted of 8 row × 12 plants (each hole has 3 seedling) with a planting density of 30 × 20 cm; B3: each plot consisted of 8 row × 24 plants (each hole has 3 seedling) with a planting density of 30 × 10 cm; C3: each plot consisted of 12 row × 24 plants (each hole has 3 seedling) with alternate 10 cm and 30 cm for row spacing, and 10 cm for plant distance. Data presented as the means with standard deviations were obtained from 24 plants of 2 years. The letter A/B/C, a/b/c represent significant difference at $P < 0.01$ and $P < 0.05$, respectively, based on ANOVA.

Table 3 Agronomic performance of Kongyu13, SSSL–*sd1–GKAR* and SSSL–*sd1–d* under three different nitrogen fertilizer conditions in 2019

	100 kg N/ha			150 kg N/ha			200 kg N/ha		
Traits	Kongyu 131	SSSL–*sd1–GKAR*	SSSL–*sd1–d*	Kongyu 131	SSSL–*sd1–GKAR*	SSSL–*sd1–d*	Kongyu 131	SSSL–*sd1–GKAR*	SSSL–*sd1–d*
PH (cm)	73.1 ± 8.3^{A}	60.7 ± 2.3^{B}	45.9 ± 2.4^{C}	72.4 ± 1.6^{A}	55.2 ± 2.2^{B}	43.9 ± 1.2^{C}	71.5 ± 2.7^{A}	55.3 ± 2.3^{B}	42.8 ± 2.5^{C}
LI	5.16 ± 1.33^{Aa}	4.16 ± 0.62^{Ab}	2.50 ± 0.50^{Bc}	4.95 ± 0.83^{Aa}	3.62 ± 1.36^{Ab}	2.02 ± 0.55^{Bc}	3.74 ± 1.17^{A}	2.38 ± 0.25^{B}	2.01 ± 0.59^{B}
ETP	23.6 ± 8.3	23.0 ± 4.3	22.8 ± 3.6	28.7 ± 3.8^{B}	32.1 ± 3.9^{AB}	35.7 ± 4.6^{A}	32.2 ± 3.5^{B}	31.0 ± 4.4^{B}	37.9 ± 5.8^{A}
GNP	105.8 ± 17.2^{Ab}	125.3 ± 17.0^{Aa}	76.2 ± 14.4^{Bc}	106.0 ± 22.4^{A}	112.9 ± 20.3^{A}	81.2 ± 10.4^{B}	107.2 ± 16.1^{A}	114.6 ± 21.3^{A}	84.5 ± 20.5^{B}
SSR (%)	94.3 ± 3.7	95.5 ± 2.4	93.9 ± 4.0	91.1 ± 4.9^{ABb}	96.2 ± 1.7^{Aa}	88.6 ± 6.0^{Bb}	89.9 ± 8.7	94.6 ± 2.3	90.5 ± 10.1
HGW (g)	2.33 ± 0.04^{A}	2.35 ± 0.05^{A}	2.23 ± 0.04^{B}	2.49 ± 0.09^{Aa}	2.45 ± 0.07^{ABa}	2.38 ± 0.08^{Bb}	2.47 ± 0.09^{A}	2.48 ± 0.05^{A}	2.35 ± 0.11^{B}
YP (g)	33.52 ± 7.97^{A}	34.74 ± 7.30^{A}	23.41 ± 5.00^{B}	51.20 ± 4.44^{A}	52.18 ± 5.14^{A}	41.57 ± 6.97^{B}	53.76 ± 6.40^{a}	53.86 ± 6.51^{a}	43.80 ± 8.88^{b}

ETP, effective tillers per plant; GNP, grain number per panicle; HGW, hundred grain weight; LI, lodging index; PH, plant height; SSR, seed setting rate; YP, yield per plant. Three fertility levels were set at B3 cultivation density with actual nitrogen application of 100 kg, 150 kg and 200 kg per hectare, respectively. Data presented as the means with standard deviations were obtained from 12 plants. The letter A/B/C, a/b/c represent significant difference at $P < 0.01$ and $P < 0.05$, respectively, based on ANOVA.

the other genes within the intrograted fragment impacted the plant yield. The 374 kb target segment between M1 and M5 carrying *sd1–GKAR* contained unidentified genes can possibly interact with *sd1*. It was also reported that the impact of *sd1* significantly reduced the chances of detecting other QTLs (Zhang *et al.*, 2020). The populations without *sd1* segregation were preferred for isolating novel plant height QTLs (Liu *et al.*, 2018).

A few recent studies reported the attempt to create novel *sd1* alleles in different rice varieties by genome editing technology (Biswas *et al.*, 2020; Hu *et al.*, 2019). In this study, we developed four CRISPR mutants in Konyu131 background with edited *SD1* gene at the corresponding adjacent region of the SNPs in the *sd1–GAKR* and *sd1–d* alleles. The resulting alleles were all regarded as loss of function as plant height was comparable among the CRISPR mutants and SSSL–*sd1–d*, but significantly lower than both SSSL–*sd1–GKAR* and Konyu131. Therefore, it can be concluded the plant height associated gene was confirmed to be *SD1* at *qKD1* locus.

There was no significant difference in plant height among the four homozygous T_1 knockout mutant lines simulating the *sd1–GKAR* allelic variation, but the plant height was significantly reduced by about 13% compared with that of SSSL–*sd1–GKAR*. There was no significant difference in plant height among the three homozygous T_1 knockout mutant lines that simulated the *sd1–d* allelic variation and SSSL–*sd1–d* (Figure S4). Comprehensive analysis of plant height of these mutant lines showed that although different editing targets were selected according to natural allelic variations, the plant height of the mutants were all similar to that of SSSL–*sd1–d*; that is, after gene editing, the wild-type *SD1* gene almost completely lost its function. We selected GKAR–T_1-1 and IR64–T_1-1 to investigate lodging index and yield-related traits, and the results also showed that lodging index and yield-related traits of gene-editing lines and SSSL–*sd1–d* were more similar (Table S1). These results demonstrated that it is still technically difficult to use gene editing technology to specifically edit one allele type of a gene into another. Gene-editing technology is mainly used to create new alleles. Previous report also showed that the CRISPR/Cas9 system is effective in producing homozygous mutants for functional analysis, but its application for successful rice breeding may be not as precise as expected and still needs more accurate molecular characterization and generations of screening (Biswas *et al.*, 2020).

The mutations generated by CRISPR/Cas9 technology are mainly base insertions and deletions, and the probability of gene fragment replacement is small. Therefore, CRISPR/Cas9 technology is mainly used for site-directed gene knockout in rice, generally for the knockout of some negative regulatory genes to achieve the purpose of improving related traits (Shen *et al.*, 2017; Zhang *et al.*, 2018). The CRISPR/Cas9-derived cytosine base editing (CBE) system and adenine base editing (ABE) system established in recent years can achieve base site-directed substitutions at target sites C to T, G to A and A to G, T to C, respectively (Gaudelli *et al.*, 2017; Komor *et al.*, 2016). The more precise base editing technology has been successfully applied to the creation of rice gene point mutations (Hua *et al.*, 2018; Lu and Zhu, 2017; Shimatani *et al.*, 2017; Zong *et al.*, 2018), which can complement the defect of low efficiency of gene fragment replacement in CRISPR/Cas9 system to a certain extent. In terms of *SD1*, the encode enzyme activity requires further study to investigate possibilities of control plant height by base editing to reduce enzyme activity to the desired levels. We also developed an advanced backcross population to explore the QTL for plant height by high-resolution mapping. The *SD1* complementary lines of map-based cloning also confirmed *SD1* as the causal gene for plant height variation. According to the five markers designed near *SD1*, the recombinant individuals were screened to narrow the target introduced fragments and eliminate the linkage drag. During this process, several rounds of continuous selfing and backcrossing were performed coupled with background genotyping to exclude the background noise and retain the QTL as homozygous segments. Although this breeding process was lengthy, a series of advanced lines containing different alleles of high yield, high quality, nutrient efficiency and resistance to different biotic and abiotic stresses can be developed in the same genetic background. In the future, two or more favourable traits can be pyramided to meet different demands. The variety improvement strategy of overcoming the lodging resistance by precisely introducing the dwarf allele *sd1–d* into the genome of Kongyu 131 provided an insight into the molecular design and precision breeding of other crops.

Conclusions

In this study, the trait and gene-based breeding strategy of improving the existing rice varieties was established to meet the demand of agricultural production. We utilized the existing molecular biology information to explore favourable alleles from forward genetics and reverse genetics to improve the lodging resistance of Kongyu 131. The introgression of *sd1–d* allele showed excessive dwarfing phenotype with compromised yield suggesting the commonly used loss-of-function *sd1* allele in rice green revolution was not suitable for rice breeding in Kongyu 131 background. While *sd1–GKAR* allele showed improved lodging resistance without yield penalty in Kongyu 131 background. This study suggest *sd1–GKAR* allele can be adopted in *japonica* rice breeding and potentially contribute to the lodging improvement in rice industry production.

Materials and methods

Rice parent materials, genome resequencing and read alignments

Kongyu131 is an early maturing *japonica* variety with strong tillering ability and cold tolerance, which is mainly cultivated in the third accumulative temperature zone of Heilongjiang province in China. The strong lodging resistance variety, GKAR (*indica* cv.), was used as the donor to develop the backcross populations with Kongyu131 for plant height AB–QTL analysis. The late flowering *indica* variety IR64 was used as the donor to introduce *sd1–d* allele into Kongyu131. The genome-resequencing libraries of the three rice parents was conducted with Hiseq2500 sequencer. The sequencing depth for each sample was 30 fold depth. Then the clean paired-end reads were mapped to the reference genome of Nipponbare (IRGSP Build4) using the BWA (Burrows-Wheeler Aligner, version: v0.7.16) software with the command 'mem-t 10 -k 32 -M'. The unique mapped reads were retained for SNP detection with the Genome Analysis Toolkit (GATK, version v3.1). The protocol and parameter used for SNP calling was as described previously (Ma *et al.*, 2018).

Design of SNP markers

Based on the SNPs information obtained from parental resequencing and sequence alignment, SNP markers were designed to

distribute throughout the genome. Firstly, 200 bp sequences on both sides of the target SNP site were extracted according to the SNP position information, then the Primer–Blast program in NCBI (https://www.ncbi.nlm.nih.gov/) was used to design specific SNP markers. The primer length was 22–24 nt, and the length of the amplified fragment was 50–100 bp. The SNP makers were verified by high-resolution melting (HRM) analysis. In order to precisely introduce target gene and eliminate linkage drag, five specific markers (M1–M5) were designed in the vicinity of *SD1* with M1 and M2 located at 481 kb and 1.1 kb upstream, M3 within the gene, and M4 and M5 at 597 bp and 432 kb downstream, respectively (Table S2).

Population construction for AB–QTL analysis

Using Kongyu 131 as recurrent parent and GKAR as donor, a series of backcross populations including BC_3 and BC_4 generations were constructed through backcross and self-cross. Among them, one BC_3F_2 population of 86 independent lines was selected for QTL analysis of plant height. A total of 129 SNP markers evenly distributed on 12 chromosomes were used for genotyping. The QTL analysis was performed by Mapmaker/QTL 1.1b (Lincoln *et al*., 1992) at a threshold LOD of 4.0.

Fine mapping of the target QTL

In order to further isolate the functional gene in the target QTL–*qKD1* on chromosome 1, the 43 lines with genetic recombinant near the *qKD1* locus were screened from a BC_3F_4 population. Each recombinant line was seeded with 12 self-crossing progenies and the plant height was measured at maturity. Recombinant individual and progeny tests were used to narrow the interval of target gene. Based on the result of AB–QTL analysis, a heterozygous recombinant individual with reduced introduced fragment selected from the BC_3F_4 population was allowed for self-pollination to obtain 1344 BC_3F_5 population. The BC_3F_5 recombinant lines were selected to repeat the AB–QTL analysis to narrow down the interval of target gene further.

DNA extraction, PCR and HRM analysis

The DNA samples was extracted by the high-throughput plant genomic DNA preparation methods (Wang *et al*., 2013). The previously described two-step method was used for PCR amplification (Feng *et al*., 2017). The SNP genotyping was performed by high-resolution melting (HRM) analysis (Hofinger *et al*., 2009).

Selection of gene editing target sites in *SD1* and design of gRNA primer

In order to confirm the semi-dwarf QTL at *qKD1* locus is *SD1* gene, CRISPR/Cas9 was used to knock out the *SD1* gene in Kongyu 131. The online tool CRISPR-P 2.0 (http://crispr.hzau.edu.cn/CRISPR2/) was used to select the target sites in *SD1* for sgRNA design (Liu *et al*., 2017). According to the CRISPR/Cas9 system, the protospacer-adjacent motif (PAM) sequence "NGG" was identified in the sequence of *SD1*. After determining the editing target sites, 19–21 nt sequence upstream of PAM sequence was used as the forward sequence, 5′-GGCA-3′ was added to the 5′ end, and 5′-AAAC-3′ was added in front of the reverse complementary sequence. Finally, gRNA1 and gRNA2 were selected, and two pairs PCR primers were designed to amplify the DNA fragment containing the two target sites, respectively (Table S3). The full sequence context and the distance of both gRNA1 and gRNA2 target sites are shown in Figure S5.

Plasmid construction and genetic transformation

Following the previously published CRISPR/Cas9 multi-gene knockout protocol (Shan *et al*., 2013), gRNA1 and gRNA2 were cloned into two SK–gRNA intermediate vectors at *Aar* I site, respectively. The intermediate vector carrying gRNA1 was digested with *Kpn* I/*Bam*H I, and the other carrying gRNA2 with *Kpn* I/*Bgl* II. Then the intermediate vector carrying both gRNA1 and gRNA2 segment was assembled into the pC1300–Cas9 binary vector by *Kpn* I/*Bam*H I ligation. OsU3 promoter was used to drive gRNA expression. Agrobacterium-mediated transformation method was used to transform the precultivated rice calli with the resulting plasmid (pC1300–Cas9–SD1) to generate transgenic rice plants. Positive transformed plants were obtained by hygromycin screening. Genomic DNA samples were prepared from transgenic plants using Cetyl trimethyl ammonium bromide (CTAB) method. The target fragment flanking the two target sites was amplified by PCR with KOD FX DNA polymerase (Toyobo, Japan), respectively, with primer gRNA-F/Rfor Sanger DNA sequencing analysis, primer sequence is shown in Table S3.

Construction of transgenic complementary vectors

Genomic DNA sequence of *SD1* and Os01g0883900 in Nipponbare was used to design PCR primers to amplify the complete genes, including 5′ UTR the coding sequence (including introns) and 3′ UTR in Kongyu 131 with KOD-FX-DNA polymerase (Toyobo, Japan). The primer sequences are provided in Table S4. The PCR procedures are as follow: denaturation at 94 °C for 2 min; 35 cycles of denaturation at 98 °C for 10 s, annealing at 74 °C for 30 s, extension at 68 °C for 7 min; and a final extension at 68 °C for 10 min.

The amplified products were recovered from the gel and ligated to the expression vector pMDC99 at *Sac* I and *Kpn* I stie. The resulting plasmid was used to transform *E. coli* DH5α competent cells. The positive monoclonal clones were selected and the inserted fragments were verified by DNA sequencing. Finally, the recombinant plasmids carrying *SD1* and Os01g0883900 were used for plant transformation as described previously (Hiei *et al*., 1994).

Development of single segment substitution lines

To develop a single segment substitution line (SSSL) with *sd1–GKAR* introduced in Kongyu131, line BC_3F_3-621A02 was selected from BC_3F_3 population (GKAR × Kongyu 131) to backcross with Kongyu131 to exclude non-target fragments from donor in the background and shorten the target fragment. From the BC_4F_1, one individual line was selected from each generation for self-pollination until BC_4F_4. Finally, a SSSL BC_4F_4-259B04 containing *sd1–GKAR* (SSSL–*sd1–GKAR*) was selected from 96 BC_4F_4 progenies (Figure 6a).

To develop an SSSL carrying *sd1–d* in the background of Kongyu 131, line BC_5F_1-22L01 was selected the BC_5F_1 population (IR64 × Kongyu 131) With similar procedure described previously, single line was selected in each generation through 5 consecutive self-pollination. Finally, the SSSL BC_5F_6-168A01 containing *sd1–d* (SSSL–*sd1–d*) was selected from the BC_5F_6 population (Figure 6b).

Evaluation of agronomic traits

Kongyu 131, SSSL–*sd1–GKAR* and SSSL–*sd1–d* were grown in Jiamusi (46°14′ N, 130°32′ E), Heilongjiang province between

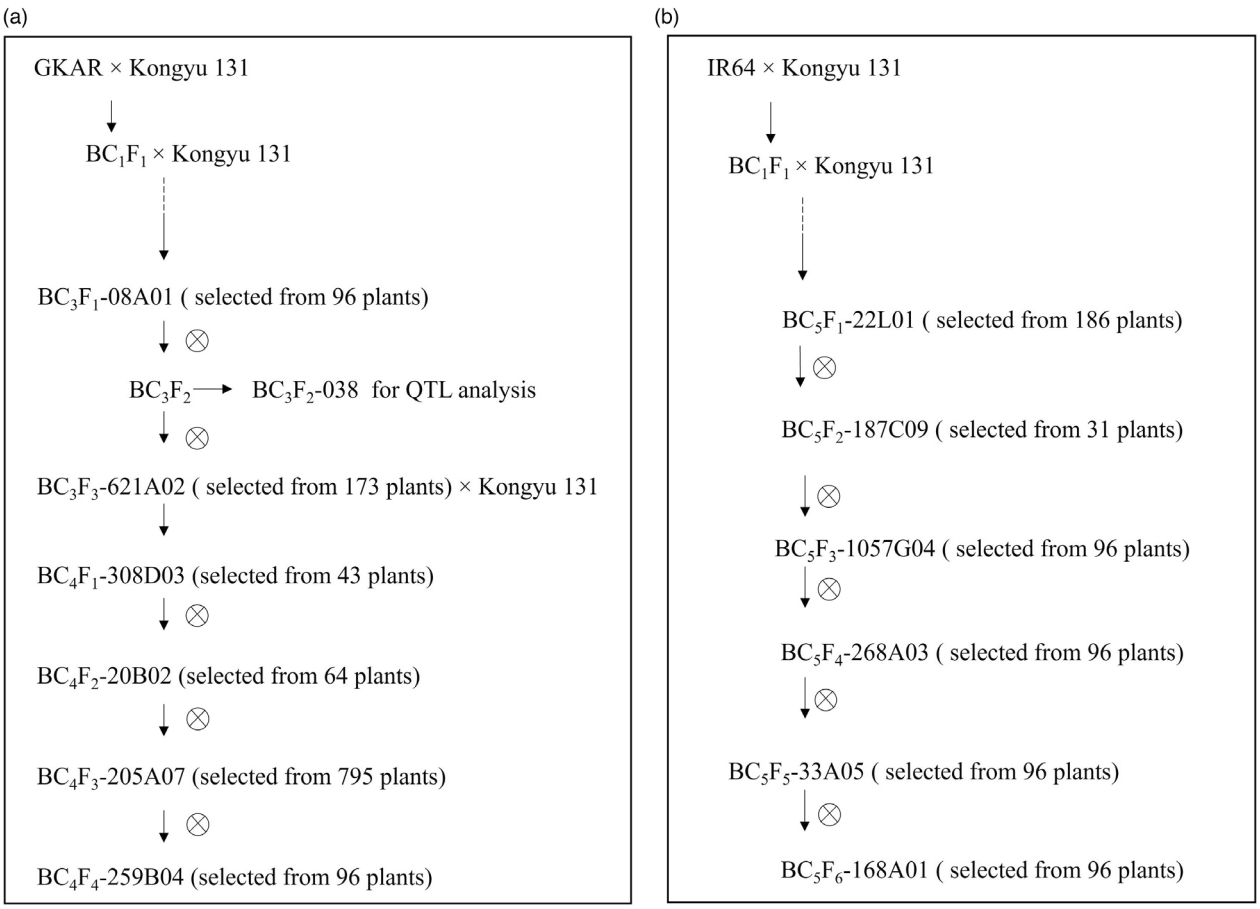

Figure 6 Development procedure of QTL mapping populations and single segment substitution lines.

2018 and 2020. In 2018, all plants were grown at the same density (A1). In 2019 and 2020, the plants were grown at four different densities (A1, A3, B3 and C3) to investigate lodging resistance and yield related traits. The four cultivation densities are as follows: A1, each plot consisted of 8 rows × 12 plants (1 seedling in each hole) with a planting density of 30 × 20 cm for each cultivar; A3, each plot consisted of 8 rows × 12 plants (3 seedlings for each hole) with a planting density of 30 × 20 cm; B3, each plot consisted of 8 rows × 24 plants (3 seedlings in each hole) with a density of 30 × 10 cm; C3, each plot consisted of 12 rows × 24 plants (3 seedling in each hole) with alternate 10 cm and 30 cm for row spacing, and 10 cm for plant distance. Three nitrogen levels (100 kg/ha, 150 kg/ha and 200 kg/ha) were applied in 2019 at B3 density following the local standard paddy field management practice, respectively.

At maturity stage, 20 plants of each line were randomly selected in the middle of each plot for the agronomic analyses including plant height (PH), lodging index (LI), effective tillers per plant (ETP), grain number per panicle (GNP), seed setting rate (SSR), hundred-grain weight (HGW) and yield per plant (YP). Plant height was measured as the distance between the neck of the highest panicle and the ground. The main stem was selected for lodging index measurement, and the tension gauge was used to apply pressure at the base 10 cm above the ground. The distance between the two fulcrum of the stem was set as 5 cm. The force when the stem was broken was recorded as the culm breaking force. LI was calculated as follows:

$$\text{LI} = \text{culm weight above breaking point (g)} \times \text{culm length above breaking point (cm)} / \text{culm breaking force (N)}.$$

Statistic analysis was carried out using SPSS with significance at $P < 0.01$ and $P < 0.05$ based on ANOVA analysis.

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Conflict of interest statement

The authors declare no conflicts of interest.

Authors contributions

SL and ZL conceived the original idea and designed the experiments; CW and XF performed the experiments, statistical analysis and wrote the manuscript; CW, XF, KL, XZ, LY, JN, WZ, RW, LW, QX and XY carried out the primers design, genotyping and evaluation of agronomic traits; QBY performed rice hybridization and field management. XF and CW revised the manuscript. All the authors read and approved the final manuscript.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 Graphical genotype of the selected lines.
Figure S2 Performance of Kongyu131, SSSL–*sd1*–*GKAR* and SSSL–*sd1*–*d* under four kinds of cultivation density.
Figure S3 Performance of Kongyu131, SSSL–*sd1*–*GKAR* and SSSL–*sd1*–*d* under different levels of nitrogen supply.
Figure S4 Plant height comparison of the mutants obtained by CRISPR/Cas9 and two SSSLs.
Figure S5 CRISPR/Cas9 gRNA target and sequence.
Table S1 Agronomic performance of two SSSLs and the mutant by CRISPR/Cas9.
Table S2 Sequences of SNP markers M1–M5 for recombination selection near SD1 gene.
Table S3 gRNA primer sequences used to edit the SD1 gene in Kongyu 131.
Table S4 PCR primer sequences used to amplify SD1 and LOC_Os01g0883900.