Using CRISPR-Cas9 to generate semi-dwarf rice lines in elite landraces

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Genetic erosion refers to the loss of genetic variation in a crop. In China, only a few original landraces of rice (Oryza sativa) were used in breeding and these became the primary genetic background of modern varieties. Expanding the genetic diversity among Chinese rice varieties and cultivating high-yielding and high-quality varieties with resistance to different biotic and abiotic stresses is critical. Here, we used the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein9(Cas9) genome editing system to edit Semi-Dwarf1 (SD1) in the elite landraces Kasalath and TeTePu (TTP), which contain many desired agronomic traits such as tolerance to low phosphorous and broad-spectrum resistance to several diseases and insects. Mutations of SD1 confer shorter plant height for better resistance to lodging. Field trials demonstrated that the yield of the new Kasalath and TTP mutant lines was better than that of the wild type under modern cultivation and that the lines maintained the same desirable agronomic characteristics as their wild-type progenitors. Our results showed that breeding using available landraces in combination with genomic data of different landraces and gene-editing techniques is an effective way to relieve genetic erosion in modern rice varieties.

Genetic erosion was proposed by Harlan1 in 1975 to describe the genetic resources after the Green revolution, which involved directional selection of semi-dwarf genes in crop plants. The semi-dwarf genes improved plant architecture in response to heavy use of nitrogen fertilizer2-3. With the modernization of agriculture and rapid urbanization, only a few modern varieties are now cultivated, in contrast to the large number of landraces used in rice production prior to the 1980s4. This has not only led to a genetic bottleneck, but has also intensified the permanent loss of many landraces5,6.

In China, the genetic erosion in rice is even more serious due to three main reasons: (1) from 1930–1960, several rice landraces (such as NanTe and Shenglixian) were dominant in the main rice-producing region of the Yangtze rice valley. (2) From 1950–1970, spontaneous Semi-Dwarf1 (SD1) mutant alleles (sd1) were derived from the main cultivars7, such as NanTe and Zhaiyeqing, or from local Taiwan varieties Dijiaowujian and Aizaizhan, and then were backcrossed into a few main cultivars. The resulting plants underwent heavy selection at the sd1 locus as well as other loci to give decreased plant height for better lodging resistance and adaptation to high Nitrogen fertilizer condition and gaining high harvest index. The main cultivars selected in the 1930s–1950s contained the sd1 mutant alleles to confer shorter plants and were used as the main breeding materials for new varieties8,9. (3) From 1970 to today, hybrid rice varieties are used to take advantage of the heterosis between two genetically distant rice varieties with semi-dwarf plant architecture and to enhance nitrogen utilization. However, increases in yield have been limited since the late 1990s. In addition, pedigree analysis showed that the parents of the hybrids had similar allelic variations and many of the same traceable linkage blocks, which when combined, may not improve yield- and resistance-related traits10,11. Together, these activities resulted in a very narrow genetic pool in the main modern Chinese rice varieties.

Relieving genetic erosion and improving the yield of modern rice varieties to satisfy food supply demands and foster sustainable development is an urgent issue. In our study, we used a clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) genome editing technique to edit Semi-Dwarf1 (SD1) and Photosensitivity5 (SE5) in the elite landraces Kasalath and TTP (TeTePu), which contain many desirable agronomic traits such as tolerance to low phosphorous12 and broad-spectrum resistance to diseases and insects13. Our results showed that precise targeting of SD1 for gene editing in Kasalath or TTP resulted

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in new lines with a semi-dwarf plant architecture, which is desired in modern rice varieties, and maintained most of the desired agronomic traits of their progenitors. We show that using gene editing on available landraces can rapidly increase genetic diversity and produce new varieties that satisfy current production requirements.

**Results**

**Kasalth and TTP sd1 mutants are resistant to lodging and show good field performance.** To rapidly create new germplasm with decreased plant height in the traditional landraces Kasalath and TTP, we constructed the CRISPR-Cas9 vector for simultaneously targeting SD1 and Photoperiod-sensitivity-5 (SE5) based on a previous study14. The guide RNA (gRNA1 and gRNA2 targeting sites were designed in the first and second exons of SD1, and the gRNA3 targeting site was in the first exon of SE5. The target sites are labeled in black lowercase letters. The protospacer adjacent motif (PAM) sequences are underlined and in red type. The two vectors used to transform rice, gRNA1 and gRNA2 were assembled into the pC1300-Cas9 expression vector for Kasalath transformation; gRNA1 and gRNA3 were assembled into the pC1300-Cas9 expression vector for TTP transformation. The internode length proportion in kasalath, sd1-1Kas, sd1-2Kas, sd1-3Kas, sd1-4Kas, and sd1-5Kas mutants. The bar in the image of whole plants represents 4 cm. Values in plant height are means ± standard deviation (±sd.), n = 10. Bars with different letters are significantly different. Statistical differences among the agronomic traits were detected by Duncan’s multiple range test (p < 0.05). Diseased leaves of 50- to 60-day-old plants from the field evaluation for resistance to blast: NPB (upper), TTP (upper the second), sd1 or sd1/se5 mutants (below). The lesion area of the TTP and TTP mutants were near 0 for all races tested.
A single base substitution, indicating that this motif is important for SD1
mutation sites were mostly detected in the gibberellin (GA) 20-oxidase (76-371) motif, varying from a deletion to spontaneous sd1 sd1 different mutation sites compared with previously reported alleles. The previously reported supported this hypothesis.

T2 progeny plants that were either homozygous sd1/sd1 or T–DNA free plants were analyzed by DNA sequencing and self-pollinated to generate T1 progeny plants. 

Table 1. Agronomic traits comparison between Kasalath and its sd1 mutant lines. Values in plant height, 
Grain number per panicle are means ± standard deviation (±sd.), n = 10. Values in tiller number, heading date means ± standard deviation (±sd.), n = 30. thousand grain weight, plot yield trial means ± standard deviation (±sd.), n = 3. Statistical differences among the agronomic traits were detected by Duncan’s multiple range test (p < 0.05).

|          | Tiller number | Primary branch | Secondary branch | Grain per panicle | Heading date | Spikelet fertility (%) | Grain Weight (g/1000) |
|----------|---------------|----------------|------------------|-------------------|--------------|------------------------|-----------------------|
| Kasalath | 13.8 ± 0.4a   | 9.9 ± 0.5a     | 41.3 ± 0.8a      | 233.5 ± 6.8a      | 84.3 ± 0.6a  | 83.2 ± 3.5a            | 18.0 ± 1.1a           |
| sd1-2Kas | 16.1 ± 0.4b   | 8.9 ± 0.2c     | 30.7 ± 0.2c      | 169.6 ± 2.1c      | 88.3 ± 0.6b  | 83.1 ± 3.5a            | 18.4 ± 0.5a           |
| sd1-3Kas | 17.0 ± 0.8b   | 9.3 ± 0.2bc    | 36.0 ± 1.6b      | 199.5 ± 5.5b      | 87.3 ± 0.6b  | 81.8 ± 3.8b            | 18.7 ± 0.4a           |
| sd1-4Kas | 16.7 ± 0.1b   | 9.4 ± 0.2ab    | 36.8 ± 0.2b      | 201.3 ± 7.9b      | 87.6 ± 0.6b  | 82.1 ± 1.9a            | 17.8 ± 1.8a           |
| sd1-5Kas | 16.9 ± 0.9b   | 9.0 ± 1.0a     | 36.1 ± 1.0b      | 200.5 ± 5.5b      | 87.3 ± 0.6b  | 84.8 ± 2.2a            | 18.1 ± 0.3a           |

2Kas, 3Kas, and 4Kas lines contain the same base deletion at the first editing target, leading to premature transcription and a similar mutant phenotype, we chose the

To test whether these four allelic variations impact yield, we measured yield-related agronomic traits including plant height, grain number per panicle, etc. The average plant height of the sd1-1Kas, sd1-2Kas, sd1-3Kas, sd1-4Kas, and sd1-5Kas lines was shorter than the WT (Fig. 1c,e), the reduction in internode length was proportional in the mutants compared to the corresponding internode length of the WT (Fig. 1d). As the sd1-1Kas and sd1-4Kas lines contain the same base deletion at the first editing target, leading to premature transcription termination and a similar mutant phenotype, we chose the sd1-2Kas, sd1-3Kas, sd1-4Kas, and sd1-5Kas lines for further research.

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Phosphorus deficiency has a detrimental impact on plant growth. Phosphatic fertilizers could relieve phosphorus deficiency under low P. We first sequenced Os07g11020/ OsGSK1 in Kasalath and its mutants and found a 14-bp deletion in the coding region. An appropriate level of seed dormancy can decrease pre-harvest sprouting, and thus improve grain yield and quality. Therefore, we analyzed whether the sd1-3Kas, sd1-4Kas, and sd1-5Kas lines have increased seed dormancy like Kasalath (Fig. 2a–c). Seed dormancy was evaluated by measuring the germination of seeds after imbibing for 6 days. We first sequenced Os07g11020/Rc in Nipponbare, Kasalath, and the sd1-2Kas, sd1-3Kas, sd1-4Kas, and sd1-5Kas mutation lines and found a 14-bp deletion in the rc from rice variety 93-11 and Nipponbare. Meanwhile, we tested the GA response of the sd1-3Kas, sd1-4Kas, and sd1-5Kas mutants and found that the mutation of sd1 can, to some degree, increase seed dormancy (Fig. 2b,c), which is similar to findings in a previous study. Together, our results suggest that the sd1 mutation in Kasalath may decrease the endogenous GA concentration and enhance seed dormancy, which may be beneficial to rice production during harvest under high temperatures and wet conditions.

Phosphorus deficiency has a detrimental impact on plant growth. Phosphatic fertilizers could relieve phosphorus deficiency, but low use efficiency of available phosphorus in rice varieties is a bottleneck and could lead to environmental consequences. Therefore, it is important to use landraces containing genes for high phosphorus use efficiency when breeding new rice varieties. A major quantitative trait locus for tolerance to phosphorus deficiency, Pup1 (also named Pstol1), was identified in Kasalath. This gene is absent from Nipponbare. In order to explore whether the sd1 mutation in Kasalath impacts phosphorus use efficiency, we conducted phenotypic analyses of Kasalath, sd1-3Kas, sd1-4Kas, and sd1-5Kas in nutrient solution with low P (0.5 mg/L), CK (10 mg/L), and high P (25 mg/L) hydropnics solution for 18 days (Fig. 3a,b). Under low P, the root lengths and surface area of sd1-3Kas, sd1-4Kas, and sd1-5Kas were increased compared with Kasalath but decline in the CK and high-P concentration (Fig. 3a,b). These results demonstrated that the sd1 mutation does not disrupt the function of Pstol1 in the Kasalath mutants under low P.

Flowering date (also known as heading date) is an important agronomic trait in rice. Photoperiod regulation is a major important factor for controlling heading date. Ghd8 (Grain Yield, Plant Height and Heading Date8) is a major quantitative trait locus associated with pleiotropic effects on grain yield, heading date, and plant height. Mutation of Ghd8 leads to earlier flowering. Heading date 1 (Hd1) represses flowering under long-day (LD) conditions and induces it under short-day (SD) conditions. Mutants of Hd1 in Kasalath exhibited no photoperiod response under LD conditions. Knockout mutants of OsGSK1 which is an orthologue of Arabidopsis BIN2 showed enhanced tolerance to cold, heat, salt, and drought stresses. OsGSK1 in Kasalath contains many single nucleotide polymorphisms (SNPs) and insertions/deletions compared with Nipponbare and 93-11. S2n can restore the sterility in indica and japonica hybrids, which is an important resource when utilizing heterosis between these rice subspecies. S2n-wide compatible gene exits in Kasalath. In order to test whether the CRISPR-Cas9 editing technique affected Ghd8, hd1, OsGSK1, and S2n and the phenotypic consequences, we sequenced Rc-HLH, Ghd8, hd1, and OsGSK in Kasalath and the sd1-2Kas, sd1-3Kas, sd1-4Kas, and sd1-5Kas mutants. In line with previous results, Rc-HLH contains a 14-bp insertion in Kasalath and its mutants. Ghd8 contained the same SNPs and

| Treatments | Plant Height (cm) | Tiller number | Grain per panicle (g) | Spikelet fertility (%) | Grain Weight (g/1000) | lodging ratio (%) |
|------------|------------------|---------------|-----------------------|------------------------|-----------------------|-----------------|
| NN         | Kasalath 152.0 ± 2.0 | 14.3 ± 0.6a | 164.7 ± 4.4a | 92.1 ± 6.1a | 16.2 ± 0.3a | 0               |
|            | sd1-3Kas 107.0 ± 1.0b | 17.7 ± 0.6a | 139.5 ± 3.5b | 85.1 ± 2.2a | 16.4 ± 0.3a | 0               |
|            | sd1-4Kas 104.7 ± 0.6b | 17.7 ± 1.2a | 135.7 ± 0.7b | 86.3 ± 1.4a | 16.7 ± 0.3a | 0               |
| NN         | Kasalath 152.0 ± 4.4a | 27.7 ± 2.3a | 152.7 ± 2.6a | 80.4 ± 5.3a | 17.0 ± 0.1a | 0               |
|            | sd1-3Kas 103.7 ± 0.6b | 32.3 ± 1.5b | 127.6 ± 2.2b | 87.3 ± 0.5a | 16.0 ± 0.3a | 0               |
|            | sd1-4Kas 106.7 ± 2.5b | 33.0 ± 3.0ab | 156.9 ± 8.7a | 81.2 ± 3.0a | 16.6 ± 1.0a | 0               |
| MN         | Kasalath 171.3 ± 4.0a | 35.3 ± 4.5a | 167.6 ± 6.0a | 81.9 ± 2.7a | 17.1 ± 0.1a | 0.8             |
|            | sd1-3Kas 108.0 ± 2.6a | 44.3 ± 2.5a | 132.8 ± 2.3b | 81.2 ± 3.0a | 16.5 ± 0.6a | 0               |
|            | sd1-4Kas 105.3 ± 0.6b | 49.0 ± 3.6b | 141.1 ± 3.3b | 85.5 ± 2.8a | 16.8 ± 0.2a | 0               |
| HN         | Kasalath 167.7 ± 5.8a | 43.7 ± 3.8a | 157.3 ± 7.4a | 65.4 ± 2.2a | 17.1 ± 0.5a | 1               |
|            | sd1-3Kas 105.0 ± 0.0a | 45.6 ± 4.9a | 126.0 ± 5.7c | 72.4 ± 5.1a | 16.6 ± 0.6a | 0.01            |
|            | sd1-4Kas 109.7 ± 2.1b | 42.6 ± 4.9a | 138.1 ± 3.6b | 73.7 ± 5.0a | 17.0 ± 0.6a | 0.02            |

Table 2. Yield-related traits comparison between Kasalath and its sd1 mutant lines under different Nitrogen treatment condition. NN: No Nitrogen, LN: Low Nitrogen- 8 kg N ha⁻¹, MN: moderate Nitrogen-14 kg N ha⁻¹, HN: high nitrogen- 20 kg N ha⁻¹. Statistical differences among the agronomic traits were detected by Duncan’s multiple range test (p < 0.05).
Figure 2. Comparison of germination among Kasalath, sd1-2\textsuperscript{Kas}, sd1-4\textsuperscript{Kas}, and sd1-5\textsuperscript{Kas}. (a) Images of caryopsis morphologies of Kasalath and the sd1 mutants. (b) Germination rates of Kasalath, sd1-2\textsuperscript{Kas}, sd1-4\textsuperscript{Kas}, and sd1-5\textsuperscript{Kas} seeds at different concentrations of GA. Germination was evaluated at 6 d after imbibition and with three samples (100 seeds/sample). (c) Germination phenotypes of Kasalath, sd1-2\textsuperscript{Kas}, sd1-4\textsuperscript{Kas}, and sd1-5\textsuperscript{Kas}. The germination phenotypes are shown at 6 d after imbibition. Values means ± standard deviation (±sd.), n > = 3. Statistical differences were detected by Duncan’s multiple range test (p < 0.05).

Figure 3. 30-day-old of root growth response under different concentration of P. (a) Total root length and surface area of Kasalath, sd1-3\textsuperscript{Kas}, and sd1-5\textsuperscript{Kas} in low-P (0.5 mg/L), CK (10 mg/L) and high-P (25 mg/L) hydroponics solution for 20 days. Error bars indicate standard error. (b) The root architecture of Kasalath, sd1-3\textsuperscript{Kas}, and sd1-5\textsuperscript{Kas} plants under different concentration of P. Values means± standard deviation (±sd), n >= 3. Statistical differences were detected by Duncan’s multiple range test (p < 0.05).
deletion in Kasalath and its mutants. *hd1* contained the same insertion mutation in Kasalath and its mutants. *OsGSK1* contained the same insertion in Kasalath and its mutants. In addition, the loss of function of *S5* was observed in Kasalath and its mutants (Fig. S7).

In the TTP mutant rice lines, we sequenced the *Pi54* gene which confer high degree of resistance to diverse isolates of *M. oryzae* and found no changes in these rice lines, which is consistent with the resistance test in the field (Fig. S8). All these results show that the *sd1* mutation in Kasalath does not have negative effects on other genes associated with many desirable agronomic traits.

In summary, The CRISPR-Cas9 editing technique can be used to more rapidly create the *sd1* mutation in desirable germplasm. Two *sd1* alleles in Kasalath and one double *sd1 se5* mutant created in this study can serve as potential materials for breeding. Creating additional *sd1* alleles in other desirable landraces could help to improve genetic diversity in rice and benefit rice production.

Materials and Methods
Plant materials and Measurements of agronomic traits. The background of transgenic plants is Kasalath or TTP and all the rice plants were grown in the paddy fields under natural conditions in Hangzhou or LingShui (China National Rice Research Institute, China). The agronomic traits were analyze after rice harvested, a total of 10 or 30 randomly chosen rice plant were use to measure plant height, grain number per panicle and tiller number, heading date.

Plasmid construction and Plant transformation. The three target sites were designed for knock out of *SD1* or *SE5* genes using the CRISPR/Cas9 system. The gRNA1SD1 (digested with Kpn I/Bgl II) and gRNA2SD1 (digested with Kpn I/Bgl II) were assembled into one intermediate vector. Similar methods were used to assemble gRNA1SE5 and gRNA3SE5 into one intermediate vector. The two intermediate vector (digested with Kpn I/Bgl II) was assembled to the pC1300-Cas9 binary vector (digested with Kpn I/BamH I), respectively. The target sequences are provided in Supplementary Table S2 in Supporting Information. The pc1300-Cas9 binary vector loading two sgRNAs was used for genetic transformation via the Agrobacterium-mediated transformation (strain EHA105) method for generating transgenic rice, according to Japonica rice and Indica rice transformation methods. Detection of mutations Genomic DNA of transgenic plants was extracted from approximately 100 mg leaf tissue of rice via the cetyltrimethylammoniumbromide (CTAB) method. PCR was conducted with KOD FX DNA polymerase (Toyobo, Japan) to amplify the fragments surrounding the three target sites. The DNA fragments were sequenced by the Sanger method and analyzed by the degenerate sequence decoding method.

Seed germination rate measurement. Seed dormancy was evaluated by germination of seed samples after-ripened for 5 days. Harvested rice was dried at 42 °C for 2 days and stored at room temperature before testing. Seeds of Kasalath and T2 of the *sd1-2*Kas, *sd1-4*Kas, *sd1-5*Kas were used in this study. Seed dormancy was evaluated by germination testing. A sample of approximately 100 well-developed seeds was distributed on filter paper in a 23 cm × 23 cm aseptic culture dishes with 150 ml distilled water at 30 °C and 100% relative humidity. An artificial climate chamber (POX-330B-22H, Life Apparatus Co., Ningbo, China) was used for the seed dormancy tests. Germination was evaluated visually by protrusion of the radicle from the hull by more than 3 mm and counted daily from day 2 to day 6 or at day 6. A test was replicated three times, and germination percentages were averaged for genetic analysis.

GA assay on 93-11, Nipponbare, Kasalath and new sd1 mutant lines. Seeds of Kasalath, T2 of the *sd1-4*Kas, *sd1-5*Kas or TTP, *sd1-1*TTP were pre-germinated in the dark at room temperature. After 3 days, germinated seeds pre-germinated in the dark at room temperature were transferred to Yoshida culture solution with 0 mM, 1 mM, 10 mM, 100 mM GA3, respectively. The solution was replaced every 3 days. The plant height and second internode length of seedlings (10 DAG) were analyzed.

Root scan of Kasalath, *sd1-3*Kas, *sd1-5*Kas grown in hydroponics. Seeds of the Kasalath and T2 of the *sd1-3*Kas, *sd1-4*Kas were pre-germinated in Petri dishes in the dark at room temperature. After 3 days, germinated seeds were transferred to Yoshida culture solution grow for 7 days. Then plants were transferred to Yoshida culture solution with low-P (0.5 mg/L), CK (10 mg/L) and high-P (25 mg/L) respectively. The solution was replaced every 3 days. The root length of seedlings (30 DAG) were checked.

N fertilizer treatments. Field experiments were conducted in the same field, HangZhou Zhejiang Province, China. The four N treatments were 0 (No N), 8 (Low N), 14 (moderate N) and 20 (high N) kg N ha⁻¹. In each treatment, N was applied at the basal, tillering and panicle initiation stages at a ratio of 4: 3: 3. Phosphorus was applied as a basal fertilizer at a rate of 5.6 kg P ha⁻¹ and K was applied equally between the basal and panicle initiation stages at 9.6 kg ha⁻¹. Kasalath, *sd1-3*Kas, *sd1-5*Kas were used in the N treatments.

Assessment of disease phenotypes with Magnaporthe oryzae. Fifty-day-old of NPB and TTP, *sd1-1*TTP, *sd1-2*TTP, *sd1-3/se5*TTP plants were inoculated with Magnaporthe oryzae spore suspension (1 × 10⁵ spores/ml). After 7 days post inoculation, disease reaction of each rice line was photographed.

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Author contributions
Qianqian conceived the project and designed the research strategies. Yongtao Cui, Xingming Hu and Anhui Feng contributed to Crispr-cas9 vector construction and transformation. Chunyan Zhao, YuZhang, JiangHu performed gene target detection. Guojun Dong, Dali Zeng, Danyin Wang, Longbiao Guo and Xingming Hu contributed to Crispr-cas9 vector construction and transformation. Qianqian, Xingming Hu, Yongtao Cui wrote the paper.

Competing interests
The authors declare no competing interests.

Additional information
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