**Effects of CRISPR/Cas9 generated *drooping leaf* (*dl*) alleles on midrib and carpel formations in *Oryza* *sativa* Nipponbare**

**Teerapong Janthabuta, Christian Tristiantob, Janejira Sakulkooc, Pongsakorn Sunvittayakulc, Anongpat Suttangkakulc,d,e, Leonardo D. Gomezf, Supachai Vuttipongchaikijc,d,e,\*, Chotipa Sakulsingharoja,\***

a Program in Genetics, Faculty of Science, Maejo University, Chiang Mai 50290, Thailand

b Department of Biology, Faculty of Mathematics and Natural Sciences, IPB University, Bogor 16680, Indonesia

c Department of Genetics, Faculty of Science, Kasetsart University, 50 Ngam Wong Wan road, Chatuchak, Bangkok 10900, Thailand

d Center of Advanced Studies for Tropical Natural Resources, Kasetsart University, 50 Ngam Wong Wan road, Chatuchak, Bangkok 10900, Thailand

e Omics Center for Agriculture, Bioresources, Food and Health, Kasetsart University (OmiKU), Bangkok, Thailand

f CNAP, Department of Biology, University of York, Heslington, York YO10 5DD, United Kingdom

**Corresponding Author:**

**Chotipa Sakulsingharoj,** Program in Genetics, Faculty of Science, Maejo University, Chiang Mai 50290, Thailand, Email: chotipa.cs@gmail.com

**Supachai Vuttipongchaikij,** Department of Genetics, Faculty of Science, Kasetsart University, 50 Ngam Wong Wan Road, Chatuchak, Bangkok 10900, Thailand, Email: [supachai.v@ku.th](mailto:supachai.v@ku.th)

**Main conclusion 30 words**

We generated drooping leaf rice mutants by CRISPR/Cas and identified two novel alleles with specific editing that allow underpinning the function of DL protein domain towards midrib and carpel formations.

**Abstract**

The *Drooping leaf* (*DL*) gene plays essential roles in regulating midrib formation and carpel specification in rice and other grass species, but the specific function of DL protein domains in different developmental processes is unclear. Analysis of different *dl* mutant alleles will allow dissecting the function of DL. Here, we generated Nipponbare rice *dl* mutants using CRISPR/Cas gene editing and identified two novel *dl* alleles with different effects on midrib formation and carpel development. Phenotypic and genotypic analysis of T0 and segregated T1 edited lines showed that while *dl-51S* allele (a 3 bp deletion and a serine deletion at position 51) reduces midrib sizes and produces normal carpels, the *dl-50LS* allele (a 6 bp deletion and a leucine-serine deletion at position 50-51) causes the lack of midribs and abnormal stigma. This result indicates that the 51-serine is important for midrib formation and the 50-leucine is essential for midrib and carpel development. These *dl* mutant alleles contribute to the *DL* gene functional analysis and to gain insights into possible modifications of leaf architecture of rice and other grass species.

Keywords: Biallelic; Development; Flower; Knockout; Leaf; Mutant; Rice

**Introduction**

Understanding the genetic control of morphology and development is important for defining plant architecture and optimizing it through breeding (Mathan et al. 2016; Wang et al. 2018). *Drooping leaf* (*DL-Os03g0215200*), a member of the YABBY-transcription factor family, emerged as one of the key genes that control both leaf-midrib formation and carpel specification in grass species (Yamaguchi et al. 2004; Strable et al. 2017; Zhao et al. 2020). The DL protein is composed of an N-terminal zinc-finger domain and a C-terminal helix-loop-helix motif (so called the YABBY domain), which play roles in the transcriptional control of downstream genes in leaf and floral development targeting in specific cell types in rice (Abiko et al. 2008; Li et al. 2011; Ohmori et al. 2011). Furthermore, *DL* was also shown to regulate awn formation in indica rice (Toriba and Hirano 2014). The molecular mechanisms by which DL regulates these three separate developmental processes are still unclear.

Disruptions of *DL* have drastic impacts on the morphology of rice plants producing drooping leaves due to the lack of midribs, ectopic formation of stamens instead of carpels and sterility due to no or abnormal carpels (Nagasawa et al. 2003). *dl* mutants with point mutations and altered amino acid sequences that showed mild *dl* phenotypes, including abnormalities from partially transformed carpels, multiple carpels, cell clustered formation and increases in the number of styles, have provided clues regarding the roles of the YABBY domain in both midrib and carpel development (Nagasawa et al. 2003; Yamaguchi et al. 2004; Ohmori et al. 2008). However, there are limited numbers of these mild *dl* mutants available, and genetic screening of *dl* mutants have proven to be laborious. CRISPR/Cas gene editing is an excellent tool to generate such a diversity of alleles that can be useful for functional studies and crop improvements (Zhang et al. 2020). In this work, we gene edited *DL* by targeting the exon 2 in Nipponbare rice using CRISPR/Cas9 and examined the *dl* phenotype in plants with edited alleles in both T0 and T1 generations. We identified *dl* phenotypes that derived from different alleles containing amino acid changes. Novel *dl* alleles and their phenotypic effects are presented here, indicating their specific function in leaf and floral development in rice.

**Materials and methods**

**Plant materials and growth conditions**

*O. sativa* var Nipponbare was used for genetic transformation. For cultivation, rice plantlets that regenerated from *in vitro* culture (T0) and germinated from seeds (T1) were cultivated under controlled conditions in 12-inches pots in greenhouse (12 h light:12 h dark at 30°C). The rice plants were fed with 46-0-0 fertilizer 7-day after transplanting, followed by 15-15-15 and 16-20-0 fertilizers at panicle formation during the reproductive stage. Four-week-old plants were used for examining the drooping leaf phenotype. Midribs were examined with a microscope (Olympus, model CX43) in leaf cross sections. Six-week-old plants were used to examine carpels, which were observed under a stereo microscope (Olympus, model SD3045).

**Vector construction and rice transformation**

AsgRNA targeting *DL* (*Os03g0215200*) was designed using CRISPR 2.0P (crispr.hzav.edu.cn). pRGEB32 (Xie et al. 2015) was used as a CRISPR/Cas9 vector system. Briefly, 5’-GGCACCTCTCCTTCCTCAGCCCG-3’ and 5’-AAACCGGGCTGAGGAAGGAGAGG-3’ oligo DNAs were firstly phosphorylated and then annealed to generate an oligo duplex before assembling with the pRGEB32 at BsaI restriction sites using the Gibson cloning (New England Biolabs). The reaction was performed by mixing 20 ng of pRGEB32, 1 μl of 10 μM phosphorylated oligo duplex, 1 μl of T4 ligase, 1 μl of T4 ligase buffer and 1 μl of BsaI-HF in a total of 10 μl volume and incubating at 37°C for 3 h. The reaction was transformed into *E. coli* strain DH5alpha, and positive clones were selected by PCR and sequencing. pRGEB32 containing *DL*-sgRNA was then transformed into *Agrobacterium tumefaciens* (strain EHA105) by electroporation.

Rice embryogenic calli were induced from mature seeds using Toki (1997) method and then transformed using *Agrobacterium* harboring the pRGEB32-DL. Briefly, calli were co-cultivated with *Agrobacterium* at 28°C for 3 days, rinsed with sterilized water 5 times and then incubated in liquid N6D media supplemented with 150 mg L-1 timentin for 5 min. Subsequently, the calli were cultivated in N6D agar media supplemented with 15 mg L-1 hygromycin B and 150 mg L-1 timentin for 2 weeks. Proliferating calli were then re-plated using fresh media for another 2 weeks before transferring to MS agar supplemented with 30 mg L-1 hygromycin B and 150 mg L-1 timentin for regeneration. The media was replaced every two weeks until plantlets were obtained.

**Genotyping of edited *dl* alleles**

Rice gDNA was isolated from leaves using a cetyltrimethyl ammonium bromide (CTAB) - based on a modified protocol from Rogers and Bendich (1994). DNA quality and quantity were analyzed using NanoDrop2000 (Thermo Scientific). The *Cas9* fragment was amplified using Cas9-F: CACCGTGGCCTATTCTGTGCTG and Cas9-R: GGGTTTTCCCAGTCACGACGTTG primers. *DL* editing sequences were amplified using Q5 DNA polymerase (New England Biolabs), gel purified and subject to sequencing analysis. The sequencing was performed by either Sanger sequencing or deep sequencing using BTSeqTM (Celemics, Seoul, South Korea) as indicated in each result section. For segregation analysis, T1 seeds were collected from the viable T0 *dl* mutant with slight drooping leaf, reduced midrib sizes and normal carpels and then grown for phenotypic and genotypic analysis. Chi-square tests were performed to verify segregation ratios.

**Results**

**Phenotypic variation of *dl* mutants generated via CRISPR/Cas9**

We designed a sgRNA (65 %GC with no off-target in the CDSs of Nipponbare genome) at the exon 2 of the *DL* gene (Fig. 1a). Twenty-four T0 plantlets were generated via *Agrobacterium*-mediated transformation using rice embryogenic calli. PCR analysis of the *Cas9* sequence revealed that 19 of 24 plantlets had the transgene (~80% transformation efficiency) (Supplementary Fig. S1). After growing for 2-3 weeks, all 19 T0 lines showed the *dl* related phenotype with some variations, indicating 100% editing (Table 1 and Fig. 1b). We found 15 T0 lines with the severe *dl* phenotype for drooping leaf, no midrib and ectopic stamens, three lines with drooping leaf, no midrib and carpels with abnormal stigma and one line with slight drooping leaf, reduced midrib sizes and normal carpels. Only the line with normal carpels was fertile. The distinct morphological changes in the midrib structure in *dl* knockout mutants are the lack of clear cells and adaxial small vascular bundle (ASV). Surprisingly, the mutant with reduced midrib size still has the ASV and clear cells, but the clear cells are smaller, and the drooping leaf phenotype is partial. High-fidelity PCR and Sanger sequencing of the target *DL* region showed that these 19 lines had differentediting in *DL*. Seventeen lines, including the fertile lines with slight drooping leaf, were bi-allelic based on overlapping chromatograms starting at 3 bp after PAM (NGG) (Fig. 1c), while the other two were homozygous for 6 bp and 5 bp deletions (Fig. 1d). The 5 bp deletion resulted in a frame-shift and thereby a gene knockout, concurrent with the knockout phenotype. Whereas the 6 bp deletion that caused deletion of leucine -serine (LS) at the position 50-51 in the DL amino acid sequence showed drooping leaves, no midrib and carpels with abnormal stigma, unlike the knockout phenotype. Interestingly, the 50-51 amino acid position is at the end of the Zinc-finger region of the DL protein. These results show different allelic effects of *dl* mutants upon midrib formation and carpel specification. In particular, the 6 bp deletion presents as a novel *dl-50LS* allele that disrupts midrib formation and impairs stigma development.

**Segregation analysis of a mild *dl* allele for fertile and slight drooping leaves**

We performed phenotypic and genotypic analysis of segregated T1 lines from the fertile bi-allelic mutant, which exhibited a mild *dl* phenotype with slight drooping leaves, reduced midrib sizes and normal carpels. From 21 T1 lines analyzed, we found 18:3 plants for slight drooping leaf/reduced midrib size/normal carpel to drooping leaf/no midrib/ectopic stamen (Table 2). A chi-square test supported the segregation ratio at 3:1 (*P* = 0.256, *n*= 2). Segregation of 3:1 ratio of the *Cas9* vector was also verified (χ*2*, *P* = 0.1014, *n*=2), indicating a single copy integration in the T0 parental line (Supplementary Fig. S1). We selected a number of T1 lines of the two distinct phenotypes for deep sequencing and found that those lines with the severe *dl* phenotype are homozygous for a 1 bp deletion (Fig. 2). In contrast, three selected lines with slight drooping leaf/reduced midrib size/normal carpel are homozygous for a 3 bp deletion. Therefore, the genotype of T0 parental line is bi-allelic for -1/-3 alleles. The 1 bp deletion causes a frameshift from the nucleotide position 346, resulting in a gene knockout. The 3 bp deletion results in a deletion of serine at position 51 of the amino acid sequence (del-51S). These data indicate that this mild *dl* phenotype with slight drooping leaves and normal carpels was derived from the *del-51S* allele, which causes the reduced size of the clear cells in the midrib while maintaining ASV. Furthermore, one of this mild phenotype is bi-allelic of 1 bp and 3 bp deletion alleles indicating that the *del-51S* is dominant over the knockout allele.

**Discussion**

In this work, we show that CRISPR/Cas gene editing generated *dl* mutants with a 100% efficiency in T0 lines (19/19 lines). Although at a low rate, homozygous lines were achieved from the T0 generation (2/19 lines). The biallelic *dl* mutants, verified by overlapping sequencing chromatograms, indicate that the editing occurred in both alleles as the knockout *dl* allele is recessive. Most of the edited lines displayed the expected severe *dl* phenotype (17/19 lines), except two T0 lines with mild *dl* phenotypes for both midrib and carpel formation. Segregation and sequence analysis of *DL* editing sites in these mild mutants provided two functional alleles for studying the regulatory control for leaf midrib and carpel development.

Previously, *dl* mutants and their underpinning alleles were identified based on the drooping leaf phenotype or homeotic transformation of a carpel into stamens (Fig. 3). *dl* knockout alleles, including *dl-sup1* to *dl-sup7*, result in the severe *dl* phenotype with drooping leaves, lack of midrib and ectopic stamen formation (Nagasawa et al. 2003; Yamaguchi et al. 2004, Li et al. 2011a, 2011b; Toriba and Hirano 2014). Recently, target mutations of *DL* using CRISPR/Cas9 also generated knockout alleles (gDL-1), and the mutants exhibited the severe *dl* phenotype (Ikeda et al. 2015). Nonetheless, mild *dl* alleles, including *dl-1* to *dl-5,* show different phenotypes. The *dl-1* spontaneous mutant has drooping leaves and abnormal carpel development, while the *dl-2* mutant shows drooping leaves and mostly normal flowers (Nagasawa et al. 2003). Together with the drooping leaf, the *dl-3* allele (unreported mutation site) causes partial transformation of carpels and generation of multiple carpels, whereas the *dl-4* allele with an amino acid replacement (E118K) shows weak carpel abnormalities (Yamaguchi et al. 2004). Finally, *dl-5* harbors an insertion in the fourth intron resulting in reduced *DL* transcript abundance, reduced midrib size and altered carpels including an increased number of styles (Ohmori et al. 2008). Here, we describe two novel *dl* alleles responsible for mild *dl* phenotypes yet distinct from the previous reports. Notably, the 6 bp deletion (*dl-50LS)* with a leucine-serine deletion is responsible for the lack of midrib and abnormal stigma, and the 3 bp deletion (*dl-51S*) with a single serine deletion is responsible for the reduced size of the clear cells resulting in reduced midrib sizes and slight drooping leaves, but normally developed carpels.

CRISPR/Cas gene editing has been used to produce rice mutants for various purposes, including improvements in agronomic traits and gene function analysis (Li et al. 2016). In this context, our mild *dl* mutants provide further functional characterization of the *DL* gene. So far, the effects of *dl* alleles were mostly explained based on transcript levels, but very little by the protein domains. The reduced transcript levels and mild phenotypes for midrib and carpel formations in *dl-1* and *dl-5* mutants indicate the dosage effect of *DL* expression (Ohmori et al. 2008). However, *DL* overexpression caused lethality at the seedling stage, presumably due to a defect in leaf development (Yamaguchi et al. 2004). Furthermore, the *dl-sup3* insertional mutant revealed a conserved regulatory region in the intron 1 that is required for transcription controls in leaf and floral organs. As a result, cis-regulatory regions for midrib formation were found within 7.4 kb of the 5’ upstream region of *DL* and in intron 1 and 2 for *DL* expression (Ohmori et al. 2011). Only *dl-4* (E118K) was able to show that the YABBY domain plays a role in both midrib and carpel development. Interestingly, the *dl* mutants generated in this work show that loss of 51-serine (*dl-51S*) at the end of the Zinc-finger domain causes reduced clear cell and midrib sizes with normal carpels, whereas loss of 50-lysine and serine (*dl-50LS*) results in no midrib and abnormal stigmas. This result demonstrates that this region, which is most likely associated with the Zinc-finger domain, is also required for the two distinct biological development of leaves and flowers.Understanding the function of DL in detail could help improve the leaf architecture for better light absorption, minimizing cultivating areas and, hence, improving grain yield without compromising the floral development.

**References**

Abiko M, Ohmori Y, Hirano HY (2008) Genome-wide expression profiling and identification of genes under the control of the *DROOPING LEAF* gene during midrib development in rice. Genes Genet Syst 83(3):237-244

Ikeda T, Tanaka W, Mikami M, Endo M, Hirano HY (2015) Generation of artificial drooping leaf mutants by CRISPR-Cas9 technology in rice. Genes Genet Syst 90(4):231-235

Li H, Liang W, Hu Y, Zhu L, Yin C, Xu J, Dreni L, Kater MM, Zhang D (2011a) Rice *MADS6* interacts with the floral homeotic genes *SUPERWOMAN1, MADS3, MADS58, MADS13,* and *DROOPING LEAF* in specifying floral organ identities and meristem fate. Plant Cell, 23(7):2536-2552

Li H, Liang W, Yin C, Zhu L, Zhang D (2011b) Genetic interaction of *OsMADS3*, *DROOPING LEAF*, and *OsMADS13* in specifying rice floral organ identities and meristem determinacy. Plant Physiol 156(1):263-274

Li M, Li X, Zhou Z, Wu P, Fang M, Pan X, Lin Q, Luo W, Wu G, Li H (2016) Reassessment of the four yield-related genes *Gn1a*, *DEP1*, *GS3*, and *IPA1* in rice using a CRISPR/Cas9 system. Front Plant Sci 7: 377

Mathan J, Bhattacharya J, Ranjan A (2016) Enhancing crop yield by optimizing plant developmental features. Development 143(18):3283-3294

Nagasawa N, Miyoshi M, Sano Y, Satoh H, Hirano H, Sakai H, Nagato Y (2003) *SUPERWOMAN1* and *DROOPING LEAF* genes control floral organ identity in rice. Development 130 (4):705-718

Ohmori Y, Abiko M, Horibata A, Hirano HY (2008) A transposon, *Ping*, is integrated into intron 4 of the *DROOPING LEAF* gene of rice, weakly reducing its expression and causing a mild drooping leaf phenotype. Plant Cell Physiol 49(8):1176-1184

Ohmori Y, Toriba T, Nakamura H, Ichikawa H, Hirano HY (2011) Temporal and spatial regulation of *DROOPING LEAF* gene expression that promotes midrib formation in rice. Plant J 65(1):77-86

Rogers SO, Bendich AJ (1994) Extraction of total cellular DNA from plants, algae and fungi. In: Gelvin Sb, Schilerpoort RA (eds) Plant molecular biology manual. Kluwer Academic Publishers, Dordrecht, pp 183-190

Strable J, Wallace JG, Unger-Wallace E, Briggs S, Bradbury PJ, Buckler ES, Vollbrecht E (2017) Maize *YABBY* genes *drooping leaf1* and *drooping leaf2* regulate plant architecture. Plant Cell 29(7):1622-1641

Toki S (1997) Rapid and efficient *Agrobacterium*-mediated transformation in rice. Plant Mol Biol Report 15(1):16-21

Toriba T, Hirano HY (2014) The *DROOPING LEAF* and *OsETTIN2* genes promote awn development in rice. Plant J 77(4):616-626

Wang B, Smith SM, Li J (2018) Genetic regulation of shoot architecture. Annu Rev Plant Biol 69:437-468

Xie K, Minkenberg B, Yang Y (2015) Boosting CRISPR/Cas9 multiplex editing capability with the endogenous tRNA-processing system. Proc Natl Acad Sci U S A 112(11):3570-3575

Yamaguchi T, Nagasawa N, Kawasaki S, Matsuoka M, Nagato Y, Hirano HY (2004) The *YABBY* gene *DROOPING LEAF* regulates carpel specification and midrib development in *Oryza sativa*. Plant Cell 16(2):500-509

Zhang Y, Pribil M, Palmgren M, Gao C (2020) A CRISPR way for accelerating improvement of food crops. Nat Food 1(4):200-205

Zhao M, Tang S, Zhang H, He M, Liu J, Zhi H, Sui Y, Liu X, Jia G, Zhao Z, Yan J (2020) *DROOPY LEAF1* controls leaf architecture by orchestrating early brassinosteroid signaling. Proc Natl Acad Sci U S A 117(35):21766-21774

**Disclosure statement**

Authors declare no conflict of interest

**Data availability statement**

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Acknowledgments**

This work was supported by the Office of the Ministry of Higher Education, Science, Research and Innovation; and the Thailand Science Research and Innovation through the Kasetsart University Reinventing University Program 2021 and Thailand Science Research and Innovation (TSRI) through Maejo University annual research grant 2021 (MJ.1-65-008). SV was supported by National Research Council of Thailand: NRCT5-RSA63002-02.

**Author contributions**

CS and SV conceived the project and designed experiments. TJ, CT, JS, PS, AS, CS and SV performed the experiments. TJ, CT, CS and SV analyzed the data. TJ, CT, CS, LG and SV prepared Figures and Tables and wrote the manuscript. All authors have approved the final version.

**Additional information**

The authors declare no competing interests.

**Abbreviations**

ASV: Adaxial small vascular bundle

CDS: coding sequence

CLV: Central large vascular bundle

CRISPR: Clustered regularly interspaced short palindromic repeats

CTAB: Cetyltrimethyl ammonium bromide

DL: Drooping leaf

PAM: Protospacer adjacent motif

sgRNA: Single guide RNA

**Table and Fig. legends**

**Table 1** Variation of *dl* phenotype for drooping leaf, midrib and carpel in *DL* edited T0 plants. The genotype of each T0 plant was determined by Sanger sequencing

**Table 2** Segregation analysis of the fertile biallelic T0 plant. Twenty-one T1 plants were observed for *dl* phenotype. The presence of *Cas9* was verified by PCR. *dl* alleles were analyzed by deep sequencing

**Fig. 1** CRISPR/Cas9 editing of *DL* gene. **a** The target site and target sequence for *DL* editing. **b** Phenotypic variation of *dl* phenotypes in T0 lines for drooping leaf, midrib formation and carpel specification (10 cm, 0.5 mm and 1 mm for scale bars, respectively). Adaxial small vascular bundle (ASV) and central large vascular bundle (CLV) are indicated. A zoomed up view shows a presence of ASV in the wild type and reduced midrib, but not in no midrib lines. **c** Chromatograms of Sanger sequencing at the editing site of wild type and bi-allelic mutants. **d** The sequences of 5 bp and 6 bp deletions in homozygous mutants. Amino acid sequences were deduced from the edited *DL* sequences

**Fig. 2** Sequence analysis of segregated T1 lines derived from the slight drooping leaf, reduced midrib and normal carpel. The nucleotide positions are indicated based on the *DL* primary transcript, and the deduced amino acid positions are indicated based on the gene coding sequence. Numbers in brackets indicate base deletions

**Fig. 3** Summary of *dl* mutant alleles reported in literature. Triangles indicate DNA insertions. Arrows indicate point mutations. Black boxes represent 5’ and 3’-UTRs, and Gray boxes represent exon regions. *dl-50LS* and *dl-51S* are alleles identified in this work

**Supplementary Fig. S1** PCR genotyping of the *Cas9* coding sequence (1,154 bp) in T0 (**a**) and T1 (**b**) editing lines. Lane M represents a ladder with indicated sizes, N represents negative controls using water as template, and P represents positive controls using the Cas9 expression vector as a template

**Table 1** Variation of *dl* phenotype for drooping leaf, midrib and carpel in *DL* edited T0 plants. The genotype of each T0 plant was determined by Sanger sequencing

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Genotype | *dl* phenotype | | | Total T0 plants |
| Drooping leaf  Lack midrib  Ectopic stamen | Drooping leaf  Lack midrib  Carpel without stigma | Slight drooping leaf  Reduced midrib  Normal carpel (fertile) |
| Biallelic | 14 | 2 | 1 | 17 |
| 6 bp deletion | - | 1 | - | 1 |
| 5 bp deletion | 1 | - | - | 1 |
| Total T0 plants | 15 | 3 | 1 | 19 |

**Table 2** Segregation analysis of the fertile biallelic T0 plant. Twenty-one T1 plants were observed for *dl* phenotype. The presence of *Cas9* was verified by PCR. *dl* alleles were analyzed by deep sequencing

| T1 lines | Slight drooping leaf  Reduced midrib  Normal carpel | Drooping leaf  Lack midrib  Ectopic stamen | *Cas9*  vector | Genotype |
| --- | --- | --- | --- | --- |
| 1 | + |  | + | ND |
| 2 | + |  | + | ND |
| 3 | + |  | - | 3-bp deletion |
| 4 | + |  | + | ND |
| 5 | + |  | + | 3-bp deletion |
| 6 | + |  | + | ND |
| 7 | + |  | + | ND |
| 8 | + |  | + | ND |
| 9 | + |  | - | 1 and 3-bp deletion |
| 10 | + |  | + | ND |
| 11 | + |  | + | ND |
| 12 | + |  | + | ND |
| 13 | + |  | + | ND |
| 14 | + |  | + | ND |
| 15 | + |  | + | ND |
| 16 | + |  | + | 3-bp deletion |
| 17 | + |  | + | ND |
| 18 | + |  | + | ND |
| 19 |  | + | + | ND |
| 20 |  | + | + | 1-bp deletion |
| 21 |  | + | + | 1-bp deletion |

ND: not determined

Graphical user interface

Description automatically generated

**Fig. 1** CRISPR/Cas9 editing of *DL* gene. **a** The target site and target sequence for *DL* editing. **b** Phenotypic variation of *dl* phenotypes in T0 lines for drooping leaf, midrib formation and carpel specification (10 cm, 0.5 mm and 1 mm for scale bars, respectively). Adaxial small vascular bundle (ASV) and central large vascular bundle (CLV) are indicated. A zoomed up view shows a presence of ASV in the wild type and reduced midrib, but not in no midrib lines. **c** Chromatograms of Sanger sequencing at the editing site of wild type and bi-allelic mutants. **d** The sequences of 5 bp and 6 bp deletions in homozygous mutants. Amino acid sequences were deduced from the edited *DL* sequences

****

**Fig. 2** Sequence analysis of segregated T1 lines derived from the slight drooping leaf, reduced midrib and normal carpel. The nucleotide positions are indicated based on the *DL* primary transcript, and the deduced amino acid positions are indicated based on the gene coding sequence. Numbers in brackets indicate base deletions



**Fig. 3** Summary of *dl* mutant alleles reported in literature. Triangles indicate DNA insertions. Arrows indicate point mutations. Black boxes represent 5’ and 3’-UTRs, and Gray boxes represent exon regions. *dl-50LS* and *dl-51S* are alleles identified in this work



**Supplementary Fig. S1** PCR genotyping of the *Cas9* coding sequence (1,154 bp) in T0 (**a**) and T1 (**b**) editing lines. Lane M represents a ladder with indicated sizes, N represents negative controls using water as template, and P represents positive controls using the Cas9 expression vector as a template