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Genome-edited ATP BINDING CASSETTE B1 transporter SD8 knockouts show optimized rice architecture without yield penalty

Dear editor,

In the 1960s, the use of semi-dwarf rice and wheat varieties ushered in the "Green Revolution," leading to reduced lodging and increased harvest index. In rice, essentially all modern semi-dwarf varieties carry a specific null mutation or weak alleles of *Semi-Dwarf1 (SD1)*, which encodes a GA20-2 oxidase in the gibberellin biosynthetic pathway (Monna et al., 2002; Sasaki et al., 2002; Spielmeyer et al., 2002). In addition to gibberellins, other plant hormones such as brassinosteroids, strigolactones, and auxin also function in reducing rice height (Ferrero-Serrano et al., 2019). However, many dwarf or semi-dwarf mutants have not been widely used in rice-breeding programs because they adversely impact grain yield (Ferrero-Serrano et al., 2019). Moreover, the flag leaf has a higher photosynthetic capacity than lower canopy leaves, which allows for greater interception of light. Rice yield is closely related to the flag leaf because it contributes about 50% of the assimilates used to fill the grain with starch (Dong et al., 2018). Crops with erect flag leaves can grow at higher plant densities without compensatory reductions in photosynthesis, leading to increased grain yield. Therefore, dwarfing and leaf erectness have been breeding targets for several decades, as components of ideal plant architecture. Identification of genes that moderately reduce rice height (semi-dwarfing) and optimize rice architecture without yield penalty is still highly desirable.

Using data from a previous genome-wide association study, we analyzed single-nucleotide polymorphisms (SNPs) associated with rice height in the 3000 rice genomes dataset (Alexandrov et al., 2015; Wang et al., 2018) and successfully identified one predicted open reading frame, *Semi-Dwarf in chr8* (*SD8*, *LOC_Os08g45030*), in a 50-kb interval (28,270,000–28,280,000) of chromosome 8 (Figure 1A). Through phylogenetic analysis, we found that *SD8* encodes a putative ortholog of *Arabidopsis thaliana* ATP Binding Cassette B1 (ABCB1)/P-glycoprotein1 (Noh et al., 2003; Geisler et al., 2005). To investigate the biological functions of *SD8* in rice, we used CRISPR-Cas9-mediated gene editing to obtain two knockout (KO) lines in the Nipponbare (NIP) background (Figure 1B). Phenotypically, *sd8-1* (one-bp insertion mutant) and *sd8-2* (two-bp deletion mutant) plants had moderately reduced height due to shorter internode lengths, as well as a smaller flag-leaf angle, and thus displayed optimized plant architecture (Figures 1B–1D; supplemental Figure 1). *sd8* mutant phenotypes could be rescued in transgenic complementation lines (supplemental Figure 2). Notably, there were no significant phenotypic differences between NIP and the two *sd8* mutants in seven yield-related traits (supplemental Figures 2E–2I and 3). Because of the desirable possibility that the combination of semi-dwarf height and leaf angle in *sd8* could increase production yields under dense planting, we investigated yields of NIP and *sd8-1* in paddy-field plots at two planting densities. In the high-density plots (560,000 plants/ha), *sd8-1* mutants and NIP plants showed yield increases of ~20.6% and ~10%, respectively, compared with those grown in low-density plots. There was no significant difference in yield between genotypes grown in the low-density plots (280,000 plants/ha) (Figures 1E and 1F; supplemental Figure 4). Collectively, these data revealed that loss of *SD8* function could optimize rice architecture by reducing plant height and flag-leaf angle without yield penalty and that *SD8* KOs may even have the potential for increased yield under high-density planting.

Consistent with *sd8* mutant phenotypes, β-glucuronidase reporter assays and quantitative real-time PCR indicated that *SD8* was primarily expressed in the internode (Figure 1G; supplemental Figure 5A). *SD8* also showed differences in expression among seven *japonica* and *indica* cultivars (supplemental Figure 5B). We observed that SD8 was localized in the plasma membrane (supplemental Figure 5C). In plants, ABCB1 homologs are known to mediate cellular efflux of indole-3-acetic acid (IAA) and to regulate polar auxin transport (Multani et al., 2003; Noh et al., 2003; Geisler et al., 2005). We therefore measured the endogenous IAA content in NIP, *sd8-1*, and *sd8-2* seedlings. IAA levels were significantly lower in *sd8* than in NIP seedlings (Figure 1H). Moreover, we found that the shortened plant height and reduced leaf-angle phenotypes of *sd8* mutants could be rescued by applying exogenous IAA (supplemental Figure 6). Consistent with the observed reduction in auxin concentration, *sd8* mutants had reduced expression of genes in the auxin signaling pathway, including *OsPIN1a/1b/2* and *OsIAA3/9/20* (Figure 1I).

To further investigate whether *SD8* modulated auxin transport in rice, we measured IAA flux speed in NIP and *sd8-1* seedlings. IAA efflux and influx currents were significantly lower in *sd8-1* than in NIP, both with and without IAA treatment (Figure 1J), suggesting that loss of *SD8* function affected IAA flux currents. In addition, we used a previously reported assay to measure auxin acquisition in the IAA-sensitive yeast strain *yap1-1* (Yang et al., 2020) and found that SD8 indeed promoted IAA accumulation in yeast, resulting in a stronger suppression of IAA-induced growth (Figure 1K).

To determine whether *SD8* had similar biological functions and loss-of-function mutant phenotypes in diverse rice varieties, we

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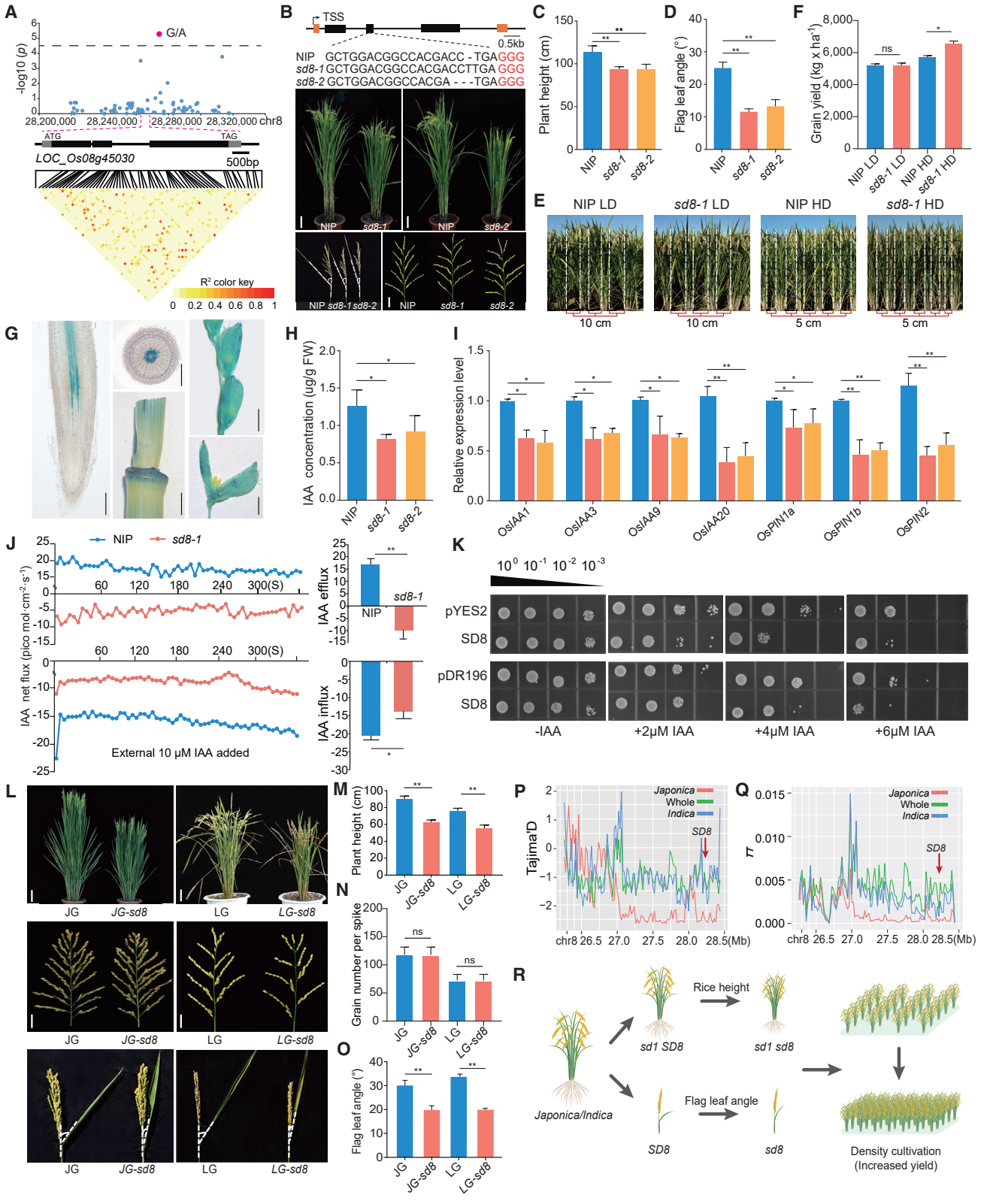

Figure 1. *SD8* knockouts showed reduced plant height and flag-leaf angle without yield penalty.

(**A**) Identification of a putative open reading frame on chromosome 8 (*LOC_08g45030*) associated with plant height based on re-analysis of SNPs in the 3000 rice genomes dataset (Alexandrov et al., 2015).

(legend continued on next page)

created CRISPR-Cas9-edited *SD8* KO mutants in two key elite cultivars in the *japonica* background, Jingeng818 (JG) and Longgeng31 (LG). Similar to the *SD8* KO plants in the NIP background, we observed a remarkable decrease in height and flag-leaf angle in *JG-sd8* and *LG-sd8* but no differences in the examined yield-related traits (Figures 1L–1O; supplemental Figure 7). We detected a considerable decrease in IAA content in these KO lines, and auxin-responsive gene expression was reduced in *JG-sd8* and *LG-sd8* (supplemental Figure 8). We also knocked out *SD8* in the *indica* rice cultivars 93-11, YexiangB (YX), Nongxiang32, and Yuzhenxiang. Similar to the *SD8* KO plants in the *japonica* background (NIP, JG, and LG), the KO lines in *indica* backgrounds also exhibited semi-dwarf phenotypes (Figure S9) and significant decreases in IAA content (Figure S10). Together, these results showed that loss of *SD8* function in different backgrounds could indeed reduce rice height and flag-leaf angle, suggesting an essential role for *SD8* in the optimization of rice architecture.

Analyses of SNPs and haplotypes (Haps) have become a major strategy for understanding evolutionary relationships and phenotypic variations, and these methods have breeding applications in rice (Wang et al., 2018). In the 3000 rice genomes dataset (Alexandrov et al., 2015), we identified 14 Haps using 16 SNPs in *SD8* (supplemental Figure 11A). The Hap frequencies differed significantly between the *indica* and *japonica* subspecies (supplemental Figures 11B and 11C). Next, we revealed significant differences in rice height among the top five most frequent Haps; Hap4 showed a significantly lower mean height than the other four Haps (supplemental Figure 11D). Based on Hap frequencies in *SD8*, we found that the *japonica* population had significantly higher Tajima's D and π (nucleotide diversity) values than the *indica* population in the ~2-Mb region flanking *SD8* (Figures 1P and 1Q). These data indicated that *SD8* has undergone strong balancing selection in the *japonica* subpopulation, suggesting that there is considerable potential for using *SD8* to balance increased productivity and reduced height.

The discovery of the semi-dwarfism gene *SD1* enabled the introduction of dwarfism to breeding programs in the 1960s, a major scientific advance for the rice Green Revolution. *SD1* has undergone significant artificial selection in *japonica* and *indica rice* cultivars (Asano et al., 2011), and different mutant alleles of *SD1* have been used separately for rice breeding in the *japonica* or *indica* background to produce semi-dwarf cultivars. The *indica* cultivars contain loss-of-function *SD1* mutations, and the *japonica* cultivars contain weak alleles. Based on previous reports, *japonica* cultivars NIP and LG contain weak *sd1* mutant alleles (*sd1-EQ type*), and *indica* cultivars YX and 9311 are considered to contain loss-of-function mutant alleles (s*d1-d* allele type for YX, and dwarf *sd1-9311* type for 9311) (Asano et al., 2011; Wu et al., 2018). Despite the many advantages of *sd1* as a source of dwarfism and lodging resistance, its widespread use has been revealed to have other associated negative effects. It has been reported that mutation of *SD1* has negative effects on spikelet number per panicle, panicle length, and branch number, eventually resulting in reduced yield (Murai et al., 2002; Su et al., 2021). Like mutation of *SD1* in modern *indica* and *japonica* cultivars, we propose that genome editing of *SD8* may have similar potential for reducing the height of *indica* and *japonica rice* cultivars (Figure 1Q). In addition, *sd8* mutation could also reduce flag-leaf angle without yield penalty. Thus, we believe that *SD8* could be an alternative dwarfing gene for rice-breeding programs and that it has more potential to further reduce rice height or even increase yield under high-density planting. The application of sophisticated genome-editing technology to *SD8* enabled us to develop sustainable rice varieties with optimized architecture and without yield penalty. This approach has the potential to revolutionize direct-seeding strategies for green-agriculture cultivation of rice.

The *Arabidopsis abcb1* mutant does not show a dwarf phenotype, in contrast to the semi-dwarf and reduced flag-leaf-angle phenotypes of *sd8* in rice (Noh et al., 2003; Geisler et al., 2005). Although mutation of ABCB1 homologs in the monocots maize (*br2*) and sorghum (*dw3*) causes a severe dwarf phenotype, grain yield is also severely reduced (Multani et al., 2003), which may hinder the application of ABCB1 homologs to the breeding of semi-dwarf plants. It will be crucial to determine whether ABCB1 homologs have conserved functional effects on auxin transport but different KO phenotypes in various plant lineages.

(**B**) Gross phenotypes of *SD8* KO lines in the Nipponbare (NIP) background obtained using CRISPR-Cas9 gene editing. Top panel: mutation sites in the two knockout lines (*sd8-1* and *sd8-2*). Bottom panels: height, flag-leaf angle, and panicle morphology in NIP, *sd8-1*, and *sd8-2* plants.
(**C** and **D**) Comparison of plant height (**C**) and flag-leaf angle (**D**) between NIP, *sd8-1*, and *sd8-2* plants.
(**E**) Representative NIP and *sd8-1* plants grown under different planting densities. Seeds from NIP and *sd8-1* were grown at high (5 × 20 cm) and low (10 × 20 cm) planting densities.
(**F**) Grain yield of NIP and *sd8-1* plants grown at high and low planting densities. ns, not significant. *$p < 0.01$ (Student's *t*-test).
(**G**) Glucuronidase staining in roots of 7-day-old seedlings, internodes at the early heading stage, glumes at the early heading stage, and glumes at the late heading stage. Scale bars: 1 mm.
(**H**) Gas chromatography–mass spectroscopy analysis of endogenous free IAA concentrations in NIP, *sd8-1*, and *sd8-2* seedlings.
(**I**) Relative expression levels of *OsIAA1/3/9/20* and *OsPIN1a/1b/2* in aerial tissues of 3-week-old NIP, *sd8-1*, and *sd8-2* seedlings.
(**J**) Time course analysis of IAA efflux and net influx in the primary root meristem of 7-day-old NIP and *sd8-1* seedlings as measured continuously for 5 min by the scanning ion-selective electrode technique. IAA influx was measured in the presence of 10 μM exogenous IAA. Columns represent the mean net influx rates averaged over the entire 5-min observation window (±SE, n = 6–10 plants). *$p < 0.05$ (one-way analysis of variance).
(**K**) *SD8* functionality assays for auxin acquisition in the IAA-sensitive yeast strain *yap1-1*. The growth status is shown for *yap1-1* cells expressing empty vectors (pYES2 and pDR196) and *SD8* on SD-U medium without uracil supplemented with 2, 3, 4, or 6 μM IAA. Serial dilutions (1:10) of yeast cells were spotted onto SD-U solid medium containing 2% galactose or glucose, then incubated at 30°C for 4 to 6 days.
(**L**) Gross phenotypes of *SD8* KO lines in the Jingeng818 and Longgeng31 backgrounds.
(**M**–**O**) Quantitative analysis of plant height and flag-leaf angle in wild-type and *SD8* KO lines in the Jingeng818 and Longgeng31 backgrounds.
(**P** and **Q**) Tajima's D and nucleotide diversity (π) values for a ~2-Mb genomic region flanking *SD8* in the 3000 rice genomes dataset.
(**R**) A model for loss of *SD8* function with and without *SD1* in which plant height is reduced but yield is increased under high-density planting.

SUPPLEMENTAL INFORMATION

Supplemental information is available at *Plant Communications Online*.

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AUTHOR CONTRIBUTIONS

X.G., S.Y., and L.Y. supervised the project. R.Q., P.Z., and Q.L. performed most of the experiments. Y.W. and W.G. analyzed the data. Z.D. and X.L. assisted with the experiments. P.Z. and X.G. wrote the manuscript. P.Z., R.Q., and X.G. revised the manuscript. All authors read and approved the final manuscript.

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