Small and round seed 5 gene encodes alpha-tubulin regulating seed cell elongation in rice

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Abstract
Seed size is an important trait in determinant of rice seed quality and yield. In this study, we report a novel semi-dominant mutant Small and round seed 5 (Srs5) that encodes alpha-tubulin protein. Lemma cell length was reduced in Srs5 compared with that of the wild-type. Mutants defective in the G-protein alpha subunit (d1-1) and brassinosteroid receptor, BRI1 (d61-2) also exhibited short seed phenotypes, the former due to impaired cell numbers and the latter due to impaired cell length. Seeds of the double mutant of Srs5 and d61-2 were smaller than those of Srs5 or d61-2. Furthermore, SRS5 and BRI1 genes were highly expressed in Srs5 and d61-2 mutants. These data indicate that SRS5 independently regulates cell elongation of the brassinosteroid signal transduction pathway.

Background
Seed size and weight are important traits for rice yield (Song and Ashikari 2008, Takeda and Matsuoka 2008). Several quantitative trait loci (QTLs) affecting seed size have been identified, namely GW2 encoding a RING-type protein that functions as an E3 ubiquitin ligase (Song et al. 2007), qSW5 encoding a novel protein with no known domains (Shoumura et al. 2008), and GS3 encoding a membrane protein with various conserved domains (Fan et al. 2006, Takano-Kai et al. 2009). Loss of GW2 and qSW5 function leads to a wider seed phenotype, and loss of GS3 function leads to a longer seed phenotype, both resulting in increased yield.

Causal genes of the small (or short) seed mutants have also been identified, namely d1 (also named RGA1) encoding the heterotrimeric G protein alpha subunit (Ashikari et al. 1999, Fujisawa et al. 1999), d11 encoding a cytochrome P450 involved in brassinosteroid (BR) biosynthesis (Tanabe et al. 2005), d2 and brd2 encoding another type of cytochrome P450 involved in BR synthesis (Hong et al. 2003, Hong et al. 2005), d61 (also named OsBRI1) encoding the BR receptor (Yamamuro et al. 2000), srs1 encoding a novel protein that has no known functional domains (Abe et al. 2010), and finally, srs3 encoding a kinesin 13 protein (Kitagawa et al. 2010). During seed formation in rice, it was demonstrated that D1 regulates cell number (Izawa et al. 2010), and SRS1 and SRS3 regulate cell length (Abe et al. 2010, Kitagawa et al. 2010). From these observations, SRS1 and SRS3 seem to affect seed size through signaling pathways other than G-protein signal transduction.

Although several genes regulating seed size have been identified, their molecular network underlying seed formation remains unclear. Here we report molecular cloning of a novel small and round seed mutant in Srs5 (Small and round seed 5). The results clearly demonstrated that Srs5 encodes alpha-tubulin and regulates cell elongation in rice seed.

Results
Characterization of the Srs5 mutant
A mutant line, Kyudai No. 37, was identified by screening of small or short seed mutants from the rice collections of Togo Field, Nagoya University, and renamed Small and round seed 5 (Srs5). Srs5 shows shorter and rounder seeds, a shorter panicle and semi-dwarf plant phenotype, compared to WT (Figure 1A-C). Additionally, F1 plants derived from a cross between WT and Srs5 plants show intermediate seed length of parents seeds (Figure 1A and 1D). From these results, we presumed that the Srs5 mutation acts as semi-dominant...
Figure 1 Srs5 mutant phenotypes. (A) Seed morphology of T65, Srs5, Srs5/SRS5, d1-1, and d61-2. Bar = 5 mm. (B) Panicle morphology of T65, Srs5, Srs5/SRS5, d1-1, and d61-2. Arrowheads indicate panicle neck nodes. Bar = 10 cm. (C) Gross morphology of T65, Srs5, Srs5/SRS5, d1-1, and d61-2. Bar = 20 cm. (D) Seed length of T65, Srs5, Srs5/SRS5, d1-1, and d61-2. Numbers on graphs indicate average seed length ± S.D. (E) Internode length relative to the total length of the culm. Schematic representation of the internode elongation pattern of T65, Srs5, d1-1, and d61-2. IN: internode.
gene. These phenotypes, short seed, short panicle, and dwarfism, are also exhibited by \textit{d1-1} and \textit{d61-2} mutants (Figure 1A-D). Comparison of internode elongation patterns among \textit{Srs5}, \textit{d1-1}, and \textit{d61-2} revealed that the internode elongation pattern of \textit{Srs5} differs from that of \textit{d1-1} and \textit{d61-2} (Figure 1E). Although \textit{d1-1} and \textit{d61-2} exhibit extremely stunted second, third, and fourth internodes, \textit{Srs5} shows equally shortened internodes (Figure 1E).

To characterize short seed phenotype of \textit{Srs5} in detail, we compared the length of the inner epidermal cells of lemmas of \textit{Srs5}, \textit{d1-1}, and \textit{d61-2} using scanning electron microscopy (SEM). The cells of \textit{Srs5} were shorter than those of the WT (Figure 2A, B, and 2F), and similar to those of \textit{d61-2} (Figure 2D and 2F), but not those of \textit{d1-1} (Figure 2C and 2F). Additionally, we estimated cell numbers by dividing lemma length (Figure 2E) and by cell length (Figure 2F). Although \textit{d1-1} had a reduced number of inner epidermal cell of the lemma, the cell numbers of \textit{Srs5} and \textit{d61-2} were not significantly different from that of the WT (Figure 2G). From these observations, we concluded that the cause of the short seed phenotype of \textit{Srs5} is reduced cell length, as in \textit{d61-2}.

\textit{SRS5} gene encodes alpha-tubulin

To map the \textit{Srs5} locus on rice chromosomes, we performed linkage analysis using F\textsubscript{2} plants derived from a cross between the \textit{Srs5} mutant \textit{(Oryza sativa. ssp.}

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**Figure 2** Comparison of inner epidermal cell length of \textit{T65}, \textit{Srs5}, \textit{d1-1}, and \textit{d61-2}. Inner epidermal cells of the lemma of WT (A), \textit{Srs5} (B), \textit{d1-1} (C), and \textit{d61-2} (D) observed by SEM. Bars = 100 μm. (E) Lemma length of the WT, \textit{Srs5}, \textit{d1-1}, and \textit{d61-2}. (F) Inner epidermal cell length of the WT, \textit{Srs5}, \textit{d1-1}, and \textit{d61-2}. (G) Estimated cell numbers of the WT, \textit{Srs5}, \textit{d1-1}, and \textit{d61-2}. Numbers in (E-G) indicate averages ± S.D.
japonica) and Kasalath (Oryza sativa. ssp. indica). Since F2 seeds show a continuous variation in seed length, short seed phenotypic expression of seed size seems to be affected by difference in genetic background between the japonica and indica subspecies, in addition to Srs5 locus (Figure 3A). From 2000 F2 plants, we obtained 13 F2 plants that produced evident short seeds in F3 progeny, indicating homozygous of Srs5 mutant gene. Linkage analysis revealed that Srs5 was located in the 1.8 Mb between chr11-7240 and chr11-9030 on chromosome 11 (Figure 3B). To further fine mapping of the gene, it was difficult to perform linkage analysis using this population, because of small number of plants with homozygous of mutant allele (small seed). For further analysis, we produced an F2 population derived from a cross between Srs5 and a Chromosome Segment Substitution Line (CSSL), SL233 that possessed the Kasalath chromosome segment of the short arm of chromosome 11 in a Koshihikari chromosome background. The phenotypes of F3 seeds obtained from these F2 plants were clearly distinguished mutant and wild types. From randomly selected 64 F2 plants, we obtained 24 WT plants (SRS5/SRS5), 36 heterozygote plants (Srs5/SRS5), and four mutant homozygote plants (Srs5/Srs5) (Figure 3C) with detecting the genotypes by using the PCR markers chr11-7240 and chr11-9030. The average seed lengths of SRS5/SRS5, Srs5/SRS5, and Srs5/Srs5 in the F2 population were 7.40 ± 0.15 mm, 6.67 ± 0.24 mm, and 5.84 ± 0.13 mm, respectively (Figure 3C). Since Srs5/SRS5 plants show seed lengths in-between those of SRS5/SRS5 and Srs5/Srs5 plants, the Srs5 mutant gene is considered to act as a semi-dominant manner (Figures 1A and 1D and 3C). The molecular markers chr11-7240 and chr11-9030 were used as selection markers for large-scale

![Figure 3 Positional cloning of the Srs5 gene.](image-url)

(A) Phenotypic distribution of F3 seed length obtained from F2 plants derived from a cross between Srs5 and Kasalath. Arrowheads indicate the average for WT, Srs5, and Kasalath. (B) Srs5 was mapped on the short arm of chromosome 11. (C) Phenotypic distribution of F3 seed length obtained from F2 plants derived from a cross between Srs5 and SL233. Arrowheads indicate the average for Srs5/Srs5 (white), Srs5/SRS5 (grey), and SRS5/SRS5 (black). (D) Linkage analysis of Srs5.
mapping (Figure 3B and 3D). We selected 664 from 5184 F2 recombinant plants that possessed chromosomal recombination within these two markers. We designed new molecular markers between chr11-7240 and chr11-9030, and 664 plants were used for high-resolution mapping (Figure 3D). The genotypes of F2 plant were confirmed by observation of the seed of F3 and F4 progeny. Finally, we obtained three recombinants within the 11 kb region including two predicted genes (RAPDB http://rapdb.dna.affrc.go.jp/) (Figure 3D). Sequence analysis revealed only one single nucleotide polymorphism (SNP) in Os11g0247300 (Figure 3D). This mutation was resulted in an amino-acid substitution from Arg to Leu in residue 308 (Figure 3D).

**Complementation test**

To confirm the SNP as causal mutation of Srs5, we performed a complementation test. The WT SR55 gene was transformed into the Srs5 mutant. The seeds of positive transgenic plants were significantly longer than the seeds of those containing empty vector (Figure 4A and 4C). The dwarf phenotype was also rescued (Figure 4B), but phenotype of transgenic plant was not completely same as wild type.

![Figure 4 Srs5 complementation test](image-url)
**SRSS expression**

Accumulation of *SRSS* mRNAs during the rice life cycle was investigated. *SRSS* mRNA accumulated in the shoot apex, young panicles, and spikelets in both the WT and the *Srs5* mutant (Figure 5A). In spikelets, accumulation of *SRSS* mRNA was higher in the *Srs5* mutant than in the WT (Figure 5A). Additionally, we compared the expression levels of *SRSS* and *BRI1* genes among *Srs5*, *d1-1*, and *d61-2* mutants. Interestingly, we detected higher accumulation of *SRSS* and *BRI1* genes in both of *Srs5* and *d61-2* mutants (Figure 5B and 5C). In *d1-1* mutant, the mRNA amounts of these genes were same level as WT (Figure 5B and 5C).

*Srs5 regulates cell elongation independently of BR signal transduction*

Since *Srs5* exhibits shorter cells, as does *d61-2*, we produced a double mutant by crossing to determine whether these two genes have epistasis. The double mutant showed shorter seed length (Figure 6A and 6C).

![Image](image.png)

**Figure 5 Expression of SRSS**. (A) Relative amounts of *SRSS* mRNA in various organs were analyzed by quantitative RT-PCR. Abbreviations: S, Shoot; R, Root; SA, Shoot apex; LB, Leaf blade; LS, Leaf sheath; LJ, Lamina joint; RA, Rachis; SP, Spikelet. (B) Relative amounts of *SRSS* mRNA in spikelet of WT, *Srs5*, *d1-1*, and *d61-2*. (C) Relative amounts of *BRI1* mRNA in spikelet of WT, *Srs5*, *d1-1*, and *d61-2*. Bars represent S.D.
and plant height than both parent mutants (Figure 6B). This result indicates that SRS5 and D61 regulate cell elongation independently during seed formation.

Discussion

In linkage analysis, we could not clearly distinguish seed size in F2 seed derived from distant cross between Srs5 and Kasalath. This was likely to be caused by background difference between indica and japonica. To overcome this, we used a chromosome segment substitution line (CSSL), which is a plant series that possesses relatively large chromosome segments of donor parent chromosomes in the recurrent parental chromosome background (Yano and Sasaki 1997, Yano 2001, Ebitani et al. 2005, Ashikari and Matsuoka 2006, Fukuoka et al. 2010). CSSLs can be used to achieve high accuracy in phenotyping in F2 populations. In fact, we could make classification of two seed size, wild and mutant type, in the F2 population derived from a cross between Srs5 and a CSSL.

Genetic analysis of the F2 population derived from a cross between Srs5 and SL233 demonstrated that the Srs5 gene act as semi-dominant manner. This semi-dominant effect was also confirmed in complementation...
test. Although the Srs5 mutants carrying WT SRS5 gene showed longer seeds than that of the plants containing empty vector, the degree of recovery was not completely same as WT (Figure 4B). The reason that the rescue by WT SRS5 gene was partial may be due to compete between WT and mutation gene products or incomplete conformation of the tubulin complex.

In this study, we demonstrated that the SRS5 gene encodes alpha-tubulin, which has been reported to be the causal gene of the rice mutation Twisted dwarf 1 (Tid1) (Sunohara 2009). The Tid1 mutation acts as a semi-dominant gene by affecting the interaction of alpha and beta tubulin. Since Srs5 was also a semi-dominant mutation, it was likely caused by incomplete conformation of the tubulin complex. Tid1 shows right helical growth, in addition to a semi-dominant dwarf phenotype. Additionally, Arabidopsis Lefty1 and Lefty2 mutations in genes orthologous to SRS5 also show semi-dominant and left helical growth (Thitamadee et al. 2002). These two mutants were gain-of-function alleles and exhibited similar twisted plant phenotypes. As the Srs5 mutant does not exhibit a twisted phenotype, different mutations in alpha-tubulin seem to lead to different phenotypes. In spikelets, higher accumulation of SRS5 mRNA was detected in the Srs5 mutant than in the WT (Figure 5A). This seems to compensate for the reduced function of alpha-tubulin protein. Higher expression of SRS5 was also detected in d61-2 but not in d1-1 (Figure 5B and 5C). Furthermore, BRII gene highly expresses in Srs5 and d61-2 but not in d1-1 (Figure 5B and 5C). These results suggest that the expression of SRS5 and BRII genes are compensated by sensing the cell elongation inhibition in the SRS5 and d61-2 mutants, although SRS5 and BRII genes regulate cell elongation independently (Figure 6D). Three other alpha-tubulin genes are present in the rice genome, and they share a high homology (Sunohara et al. 2009). In organs that exhibited no significant change in phenotype in the Srs5 mutant, these alpha-tubulins might work redundantly to maintain rice body planning.

Conclusions
Our study demonstrated that short seed mutants can be classified into two types: those with reduced cell numbers, e.g., d1, and those with reduced cell length, e.g., d61 (Figure 2A, C, and 2D). This facilitates classification of novel seed mutants. The short seed phenotype of Srs5 was demonstrated to be caused by reduced cell length, as in d61; however, the additive phenotype of the double mutant indicated that SRS5 and D61 regulate seed length via different mechanisms. To evaluate the mechanisms regulating seed length, observation of microtubule arrangement and analysis of double mutants among srs1, srs3, Srs5, and various BR mutants need to be performed.

Methods
Plant materials and growth conditions
Kyudai No. 37 was first identified at Kyushu University and maintained in Togo Field, Nagoya University. Its genetic background is unknown. A japonica cultivar, Taichung 65, was used as the WT plant. d1-1 was identified as spontaneous mutant ‘Daikoku’ and substituted its genetic background into Taichung 65 by backcrossing with Taichung 65 as a recurrent parent at Kyushu University. d61-2 was obtained by MNU treatment of Taichung 65. F2 and two parental lines were sown at the beginning of April. The seedlings of all plants were transplanted at the beginning of May into a paddy field at the Research Center for Bioresources Development in Fukui, Japan. They were then grown under natural conditions. Transgenic plants were grown in a closed greenhouse under natural sunlight. Room temperature was maintained at 30°C from 09:00 to 18:00 and 25°C from 18:00 to 09:00.

Linkage analysis of SRS5
For mapping, F2 plants derived from a cross between Srs5 (japonica) and Kasalath (indica) were used. Genomic DNA was extracted from fresh leaf tissues of 13 F2 plants that exhibited the small and round seed phenotype by the CTAB method. The genetic linkage between the Srs5 locus and molecular markers was determined using the sequence tagged site (STS) and cleaved amplified polymorphic sequence (CAPS) markers reported by the Rice Genome Program and microsatellite markers (McCouch et al. 2002). F2 plants derived from a cross between Srs5 and SL233 were used for fine mapping of Srs5 gene. Recombinant plants possessing a recombination between PCR markers chr11-7240 and chr11-9030 were screened from 5184 F2 plants. Other markers on chromosome 11 were designed by comparing the sequences of Srs5 and SL233. Information on the PCR markers used in this study is shown in Table 1. Phenotypes were determined using F3 seeds obtained from F2 plants and F4 seeds obtained from F3 plants.

Production of transgenic plants
The BAC clone containing the SRS5 gene was screened from the BAC library (constructed by the CUGI BAC/EST Resource Center) using four PCR primers, alpha-tub-5kb-up, alpha-tub-intron, alpha-tub-exon, and alpha-tub-5kb-down.

The BAC clone OSJNBA0014D2 was partially digested by Sau3AI and cloned into the BamHI site of binary vector pYLTAC7 (Liu et al. 1999) (provided by RIKEN BioResource Center). This clone contains the 7.26 kb upstream region from the transcriptional start site of the Srs5 gene and the 1.13 kb downstream region from the end of the 3’UTR region of the SRS5 gene. The
binary vector was transformed into Agrobacterium tumefaciens strain EHA105 (Hood 1993) by electroporation, and Srs5 mutants were transformed as reported previously (Ashikari et al. 2005). Srs5 mutants containing empty vectors were used as controls.

RNA isolation and RT-PCR
Total RNA was extracted using the RNeasy Mini Kit (QIAGEN, Hilden, Germany). cDNAs were synthesized from total RNA using the SuperScript III system (Invi-
tron, Carlsbad, CA, USA).

For quantification of SRS5 mRNA, real-time RT-PCR was carried out using SYBR Premix Ex Taq™ II (TAKARA Bio, Inc., Tokyo, Japan). Two primers, RT-
alpha-tub, were used to quantify Srs5 expression; a further two, RT-OsUbiquitin, were used to quantify OsLbiquitin1 expression (accession No. Os0g06814000). The Thermal Cycler Dice Real Time System (TAKARA Bio, Inc.) was used for quantification for real-time RT-
PCR.

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Competing interests
The authors declare that they have no competing interests.

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Table 1 Primer sequences used in this study

| Chr | Forward (5’→3’) | Reverse (5’→3’) |
|-----|----------------|----------------|
| chr11-7240 | GTTCAAGTCCTGCTACGTTAC | GAGGATCTATTATTTGGCA |
| RM26324 | GAGATGAGAGGAAGGTACTAG | GTTCATGTTGACATCACTAAAC |
| RM26346 | GCCGTCTGAGAATTATGG | GTATCGCTGCTGTGTTAACAA |
| RM26352+ | GCCTACCTTCAGTTAAACAA | GATAGATAAGAAGACAGC |
| SeqUP1 | GTGTGCTTCTCCAAATGTGATA | CATACATAATTCAATAG |
| chr11-7965 | GACGTGACTAAAGCTGTTT | GATTAAGCTGCTTATAGAC |
| chr11-8080 | CTCAAGTATCTGATCC | TCAAGTCTGCTTACCAAGC |
| chr11-9030 | TGCTCACGCCATCTAATGAG | TCAACATGCAACGAGTIC |
| alpha-tub-5kb-up | GTGCTCAAGATGTCATGTA | GTGCTCAAGATGTCATGTA |
| alpha-tub-intron | GCCATGATCCGTGCTGAAAT | GCCATGATCCGTGCTGAAAT |
| alpha-tub-exon | CGACAATGAGGCCACATCAT | CGACAATGAGGCCACATCAT |
| alpha-tub-5kb-down | GCTCCTTCAGCAAATCAAGA | GCCATGACCGGAGCAGGTC |
| RT-alpha-tub | ATGAGGAGTGCATCCTGAT | CAAGATCGAGGAACAGAC |
| RT-OsUbiquitin | CTTGGTGCTGTCCCGTTTC | TTCTCCAGTCGTCTACAC |

Authors’ contributions
SS carried out molecular genetic studies, expression analysis, electron microscopic analysis, and transgenic analysis, and wrote manuscript. IK carried out molecular genetic studies. TA carried out molecular genetic studies. MY carried out molecular genetic studies and wrote manuscript. HK provided all plant materials KM designed research, carried out molecular genetic studies, expression analysis, electron microscopic analysis, and transgenic analysis, and wrote manuscript. YI designed research and wrote manuscript. All authors read and approved the final manuscript.

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