**SSD1**, which encodes a plant-specific novel protein, controls plant elongation by regulating cell division in rice

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Abstract: Plant height is one of the most important traits in crop improvement. Therefore revealing the mechanism of plant elongation and controlling plant height in accordance with breeding object is important. In this study we analyzed a novel dwarf mutant, *ssd1*, of which phenotype is different from typical GA- or BR-related dwarf phenotype. *ssd1* exhibits pleiotropic defects in elongation of various organs such as stems, roots, leaves, and flowers. *ssd1* also shows abnormal cell files and shapes, which suggests defects of normal cell division in the mutant. Map-based cloning and complementation test demonstrated that the dwarf phenotype in *ssd1* mutant was caused by insertion of retrotransposon in a gene, which encodes plant-specific protein with unknown biochemical function. A BLAST search revealed that *SSD1*-like genes exist in diverse plant species, including monocots and dicots, but not fern and moss. Our results demonstrate that *SSD1* controls plant elongation by controlling cell division in higher plants.

Keywords: dwarf mutant, cell division, plant-specific protein

Introduction

Plant height, one of the most important traits in crop improvement, is related to lodging resistance, grain yield, and biomass production. Reducing plant height and conferring lodging resistance are the predominant strategies in crop improvement. Two well-known semi-dwarf genes, which have significantly contributed to the history of crop breeding, are semi-dwarf1 (*sd1*) in rice and Reduced height1 (*Rht1*) in wheat.1) Introduction of semi-dwarf varieties that possess these semi-dwarf genes, combined with application of large amounts of nitrogen fertilizer, has led to a remarkable increase in cereal production, referred to as the “green revolution”.2),3) Since these landmarks in agricultural history clearly highlighted the importance of semi-dwarf traits in crop improvement, these genes have been extensively used for breeding of high-yield and non-lodging varieties.1),4) However, a recent increase in the demand for lignocellulosic biomass for biofuels has led to a paradigm change in plant breeding. Current agricultural practices and the green revolution have significantly changed plant height to produce short, lodging-tolerant varieties with high grain yield. For dedicated bioenergy crops, however, one of the main breeding objects is to increase total biomass production, and thus the dwarf is a disadvantageous phenotype for these crops. Thus, revealing the mechanisms of plant elongation and controlling plant height in accordance with breeding objects is very important.

Analysis of dwarf mutants is a powerful method to clarify these mechanisms. To date, more than 60 dwarf rice mutants have been identified,5) and several dwarf genes have been cloned through map-based cloning.6)–9) Dwarf arises from various types of defects, but two factors responsible for drought, gibberellin (GA) and brassinosteroid (BR), have been intensely studied. Many dwarf mutants defective in the biosynthesis or perception of these phytohormones have been isolated and characterized.10)–15) As a result of intense study, typical phenotypes of rice GA- and BR-related mutants have been cata-

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loged. For example, typical rice GA-related mutants show dwarf phenotype with dark green, rough leaves, but exhibit no other abnormal morphologies except for sterility in severe mutants.\(^1\) In contrast, typical rice BR-related mutants show dwarf phenotype with other abnormal morphologies, including malformed leaves with twisted, stiff blades.\(^2\) In contrast to the GA- and BR-related dwarf mutants, little is known about mutants with phenotypes that differ from the typical GA- or BR-related phenotypes. These uncharacterized dwarf mutants may have various defects, including the division and/or elongation of stem cells, and analyzing these mutants may provide novel insights into the mechanisms of stem elongation. In fact, recent study of a high-tillering dwarf mutant has shown that a novel mechanism is involved in stem elongation in rice; that is, strigolactone, which is a carotenoid-derived, branching inhibiting hormone, and defects in strigolactone biosynthesis, or perception, result in a high-tillering dwarf phenotype.\(^3\)

To further clarify the molecular mechanisms of stem elongation in rice, we have collected dwarf mutants with phenotypes that differ from the typical GA- or BR-related mutants. These mutants were categorized into several groups according to their morphology. For example, one group has leaves with a unique rounded tip,\(^4\) another group exhibits extreme dwarf with dark and narrow leaves,\(^5\) and a third group exhibits severe dwarf with wide, stiff, dark green leaves.\(^6\) In this study, we investigated the dwarf mutant, \textit{sword shape dwarf1} (\textit{ssd1}). This mutant has defects not only in stem elongation, but also root and flower elongation. Map-based cloning revealed that \textit{SSD1} encodes a plant-specific protein with unknown biochemical function. Our results suggest that \textit{SSD1} may have a fundamental function in cell division in plants.

Materials and methods

Plant materials and growth conditions. Wild-type (WT) rice plants (\textit{Oryza sativa japonica} cv. Nipponbare) and \textit{ssd1} mutants were used in this study. These lines were grown under natural field conditions in the research field of Nagoya University, Togo, Aichi, Japan. Seeds of all lines were immersed in water for 2 days and sown in a nursery bed. One-month-old seedlings were transplanted to the paddy field at a spacing of 20 × 35 cm.

**Plasmid construction, transformation, and growth conditions.** To construct the binary vector, pBI-Hm12, containing the entire \textit{SSD1} genomic region, the BAC clone was digested with \textit{NdeI} followed by blunting the ends with \textit{T}_{4} polymerase. The 8.4-kb fragment was inserted into the \textit{SmaI} site of the pBluescript vector (Stratagene, La Jolla, CA, USA). The clone was digested with \textit{KpnI} and \textit{NotI}, after which the ends were blunted by \textit{T}_{4} polymerase. The 8.4-kb fragment was inserted into the \textit{SmaI} site of pBI-Hm12. The binary vectors were then introduced into \textit{Agrobacterium tumefaciens} strain EHA101\(^7\) by electroporation. Rice transformation was performed as described by Hiei et al.\(^8\) Transgenic plants were selected on a medium containing 50 mg/l hygromycin. Hygromycin-resistant plants were transplanted to soil and grown at 30 °C under a 16-hour light/8-hour dark photoperiod.

**RNA isolation and RT-PCR analysis.** Total RNA was isolated from various rice organs with TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The first strand cDNA was synthesized from 2 μg of total RNA using an Omniscript reverse transcription kit (Qiagen, Hilden, Germany). Semiquantitative RT-PCR analysis was performed to estimate the levels of the \textit{SSD1} gene transcript.

**Results**

Characterization of the \textit{ssd1} mutant. The \textit{ssd1} mutant was screened from the \textit{Tos17} mutant library, which is a mutant population induced by the \textit{Tos17} retrotransposon.\(^9\) In the progeny of heterozygous plants, the segregation ratio of the normal phenotype to the dwarf was 90:31, which corresponded to the expected 3:1 segregation ratio of a single recessive gene ($\chi^2 = 0.003$). The dwarf phenotype in rice is generally caused by a reduction in culm length. Based on the elongation pattern of internodes, rice dwarf mutants are classified into six types: \textit{N-}, \textit{dn-}, \textit{dm-}, \textit{d6-}, \textit{nl-}, and \textit{sh-type}.\(^10\) Of these, the \textit{dn-type} is defined by reduction in internodes length in the same proportion to the WT. \textit{ssd1} exhibited a reduction in the length of all internodes in the same proportion as in the WT, which is characteristic of the \textit{dn-type} dwarf (Fig. 2A), with severe dwarf and wide, dark green leaves (Fig. 1A, B). Culm length of the mutant at harvest was about 17 cm, whereas the WT grew to about 90 cm (Figs. 1A, B and 2A). Elongation of the seminal and crown roots
Fig. 1. Morphological characterization of the ssd1 mutant. (A) Gross morphology of Nipponbare (left) and ssd1 (right) at the ripening stage. Bar = 20 cm. (B) A close-up view of ssd1. Bar = 20 cm. (C)–(F) Morphology of flower exteriors (C), flower interiors (D), roots (E), and stamens (F) of Nipponbare (left) and ssd1 (right). Bar = 5 mm in (C) and (D), 5 cm in (E), and 1 mm in (F). (G) Morphology of pistils of Nipponbare (left) and ssd1 (middle and right). Bar = 1 mm.

Fig. 2. Lengths of panicle, internodes, and seminal roots. (A) Diagram of internode lengths of Nipponbare and the ssd1 mutant. (B) Comparison of seminal root length between Nipponbare and the ssd1 mutant. ** indicates significant differences at the 1% levels, as judged using the Student’s t test.
was also inhibited in the mutant (Figs. 1E and 2B), and the development of oral organs was also impaired in ssd1. Rice flowers are composed of four kinds of glumes, two rudimentary glumes, two empty glumes, lemma and palea, and three kinds of oral organs, two lodicules, six stamens, and one pistil. The ssd1 glumes were shorter than that of the WT (Fig. 1C, D). The ssd1 flowers also developed short anthers and stamens (Fig. 1F) and short, shrunken stigma (Fig. 1G). Some pistils developed three stigmas (Fig. 1G). These observations suggest that SSD1 has a fundamental role in cell division and/or elongation in various organs.

**Cell morphology of ssd1 leaves.** Usually, dwarf is caused by a defect in cell division and/or elongation. To clarify which defect causes dwarf phenotype in ssd1 mutant, we observed the microscopic structure of ssd1 leaves. In the WT, cell files that were well organized in a longitudinal manner were observed (Fig. 3A). In contrast, in ssd1, longitudinally arranged cells were not well organized and the cells were enlarged and distorted, leading to a disorganized cell files (Fig. 3B). Moreover, abnormality in the shape and size of cells were observed in ssd1. In the WT, only rectangular cells were observed (Fig. 3A), but trapezoidal, triangular, circular, and diamond-shaped cells were found in ssd1 (Fig. 3B, arrowheads). These abnormal cell shapes and disorganized cell arrangements are probably caused by a defect in synchronous division in these cells. In fact, the transverse division of cells was often slanted in the mutant (Fig. 3B), whereas this abnormal division pattern was not observed in the WT (Fig. 3A).

**Map-based cloning of the SSD1 gene.** To elucidate the molecular mechanism of expression of the SSD1 gene, of which dysfunction causes dwarf in the mutant, map-based cloning was performed. Previously, we reported that SSD1 was located on the short arm of chromosome 3 by linkage analysis using an F2 population derived from a cross between ssd1 and Kasalath.20) In this study, about 4,600 F3 were used for fine mapping of the SSD1 gene. The segregation in the F2 and F3 generations was apparently distorted, with the frequency of the ssd1 homozygous plant at about 6%. This is probably due to a reproductive barrier observed in the population derived from the cross between Nipponbare and Kasalath.26) Linkage analysis indicated that the SSD1 gene was tightly linked with one derived cleaved amplified polymorphic sequence (dCAPS) marker, bas46, and was localized within the 40.4-kb region between the sequence tagged site (STS) marker, S2188, and the dCAPS marker, bas41 (Fig. 4A). Cross-referencing with the Rice Annotation Project Database (RAPDB)27) showed five putative open reading frames (ORFs) in this region (Fig. 4A). Genomic DNAs corresponding to the five putative ORFs were PCR amplified from the ssd1 mutant and sequenced. Sequence comparison revealed a Tos17 insertion in an ssd1-derived gene, Os03g0302900 (Fig. 4A), while no sequence differences were found in other predicted ORFs. Therefore, Os03g0302900 was considered to be a strong candidate for the SSD1 gene.

To confirm that the Tos17 insertion in ssd1-derived Os03g0302900 is responsible for the mutant phenotype, we performed a complementation experiment. An 8.4-kb genomic DNA fragment derived from Nipponbare containing the entire Os03g0302900 sequence was introduced into ssd1 by Agrobacterium tumefaciens-mediated transformation. The dwarf
phenotype of ssd1 was rescued in all T_0 plants resistant to hygromycin, which was the selection marker used for transformation (Fig. 4B). These results confirmed that the ssd1 phenotype is caused by the insertion of Tos17 into Os03g0302900.

SSD1 encodes a plant-specific protein with unknown biological function. Sequence comparison between genomic DNA and cDNA revealed that SSD1 consists of six exons and five introns encoding 994 amino acids (Fig. 5A). ssd1 has a Tos17 insertion in exon 4 (Fig. 4A), which induces a frame shift that creates a new stop codon. Moreover, compared with WT, the mRNA level of SSD1 was significantly reduced in this mutant (Fig. 4C). Altogether these observations, this allele is likely to be a biochemical null. Basic Local Alignment Search Tool
(BLAST) analyses identified SSD1-like genes from diverse other plant species, including monocots and dicots, but not from moss (Physcomitrella patens) and fern (Selaginella moellendorffii). Three putative homologous genes, At5g26910, At3g58650, and At3g05750, which shared around 30% amino acid sequence identity with the SSD1 protein, were identified in the Arabidopsis genome. Putative SSD1 homologous genes were also identified from the genomes of Sorghum bicolor, castor bean (Ricinus communis), grape (Vitis vinifera), and western balsam poplar (Populus trichocarpa) (Fig. 5B). Database searches could not identify animal or yeast proteins that have significant similarity to SSD1. Although high similarity among diverse plant species implies that the SSD1 protein family has a fundamental function in plants, searches failed to identify any protein with known biological function in the public database.
Expression analysis of SSD1. The pleiotropic effect of the ssd1 mutation in various organs indicates that the SSD1 gene functions in all of these organs. Therefore, we studied the expression profiles in various organs. First, we performed an RNA gel blot analysis to examine expression profiles, but signals were not detected, probably due to the low expression levels of SSD1 (data not shown). Thus, we compared the expression pattern by using semi-quantitative reverse transcription (RT)-PCR. As expected, the expression of SSD1 was observed in each of the organs we tested (Fig. 6). Expression levels in the shoot apex and elongating roots were relatively higher than in other organs, whereas expression levels in the leaf blades, leaf sheaths, and flowers were relatively lower. The preferential expression of SSD1 in the shoot apex and root may correspond to the ability of each organ to carry out cell division.

Discussion

In this study, we analyzed the severe dwarf rice mutant, ssd1, screened from Tos17 mutant lines. Phenotype of ssd1 was different from well-known, typical GA- or BR-related phenotypes. Our results demonstrated that mutations in SSD1 result in pleiotropic defects in elongation of various organs, such as the stems, roots, leaves, and flowers. Compared to the WT, those organs of the ssd1 mutant were shorter (Figs. 1 and 2). ssd1 also exhibited abnormal cell files and shapes (Fig. 3B). According to these observations, the abnormal phenotype of the ssd1 mutant is probably caused by defects in cell division.

Map-based cloning revealed that SSD1 encodes a plant-specific novel protein with unknown biological function. A BLAST search disclosed that SSD1-like genes exist in diverse plant species, including monocots and dicots, but not fern and moss (Fig. 5B). The system that controls cell division by SSD1 may exist only in higher plants. The drastic and pleiotropic phenotypic effects of the ssd1 mutant and the existence of SSD1-like genes in various plant species suggest that the function of SSD1 is essential for plant cell division, but we could not identify any protein with a known biological function in the public database.

Using the WoLF PSORT programs (http://wolfpsort.org/), it was predicted that the SSD1 protein contained a putative nuclear localization signal (NLS, RKKLFSNSPEGAKLR, amino acid residues 37–53; Fig. 5A). This prediction suggests that SSD1 functions in the nucleus. This NLS is highly conserved among various plant species (data not shown), and thus this domain may have an essential role in SSD1 function.

Our group reported that mutations in the DGL1 gene resulted in a severe dwarf phenotype. Although the gross morphology is different from dgl1, the phenotype of ssd1, such as shorter stems, roots, leaves, and flower organs, is similar. dgl1 also exhibits abnormal cell files and shapes, as in ssd1. Both SSD1 and DGL1 are preferentially expressed in the organs where cell division actively occurs. DGL1 encodes rice KTN1, a 60-kD microtubule-associated ATPase katanin-like protein. Katanin is a heterodimeric protein that mediates ATP-dependent destabilization of microtubules. Mutation of the KTN1 results in aberrant microtubule orientation followed by defective cell elongation. The resemblance of phenotypes between ssd1 and dgl1 evokes the idea that both SSD1 and DGL1 control cell division through the same pathway. However, in contrast to SSD1, katanin is present in both plants and animals; therefore, cell division controlled by coordinated effect of the SSD1 and DGL1 may be a plant-specific phenomenon, or SSD1 may control cell division by a plant-specific, novel pathway that is independent of the DGL1 pathway. Our results demonstrate that SSD1 plays an important role in the regulation of cell division by an unknown pathway.

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