Positional Cloning of Rice Semidwarfing Gene, sd-1: Rice “Green Revolution Gene” Encodes a Mutant Enzyme Involved in Gibberellin Synthesis

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Abstract

A rice semidwarfing gene, sd-1, known as the “green revolution gene,” was isolated by positional cloning and revealed to encode gibberellin 20-oxidase, the key enzyme in the gibberellin biosynthesis pathway. Analysis of 3477 segregants using several PCR-based marker technologies, including cleaved amplified polymorphic sequence, derived-CAPS, and single nucleotide polymorphisms revealed 1 ORF in a 6-kb candidate interval. Normal-type rice cultivars have an identical sequence in this region, consisting of 3 exons (558, 318, and 291 bp) and 2 introns (105 and 1471 bp). Dee-Geo-Woo-Gen-type sd-1 mutants have a 383-bp deletion from the genome (278-bp deletion from the expressed sequence), from the middle of exon 1 to upstream of exon 2, including a 105-bp intron, resulting in a frame-shift that produces a termination codon after the deletion site. The radiation-induced sd-1 mutant Calrose 76 has a 1-bp substitution in exon 2, causing an amino acid substitution (Leu [CTC] to Phe [TTC]). Expression analysis suggests the existence of at least one more locus of gibberellin 20-oxidase which may prevent severe dwarfism from developing in sd-1 mutants.

Key words: rice (Oryza sativa L.); positional cloning; sd-1; cleaved amplified polymorphic sequence (CAPS); single nucleotide polymorphism (SNP)

1. Introduction

Semidwarfism is one of the most important traits in cereal crops, including rice. The rice semidwarf-1 (sd-1) gene is well known as the “green revolution gene.” This gene has contributed to the significant increase in crop production seen in the 1960s and 1970s, especially in Asia.1 This gene, originally derived from the Chinese cultivar Dee-Geo-Woo-Gen (DGWG), provides rice cultivars with short, thick culms, raises the harvest index, improves lodging resistance and responsiveness to nitrogen fertilizer, resulting high yields without affecting panicle and grain quality.1 sd-1 has been introduced by conventional breeding procedures, but the importance of this gene makes the identification of sd-1 highly desirable for the efficient production of high-yield crops via genetic engineering. Several studies have reported that sd-1 is linked to certain trait or protein loci,2–5 and to several molecular markers on chromosome 1.6–8 However, the resolution of these genetic analyses is not enough for gene identification.

Positional cloning is a strategy that uses naturally occurring genetic recombination as a means of isolating and identifying trait genes. In theory, all genes expressing observable phenotypic traits encoded on chromosomes of sexually reproducing organisms are targets with this strategy. Trait genes have already been isolated by positional cloning in Arabidopsis9 and other higher plants such as tomato10 and rice.11,12 Analyzing a large segregating population is desirable for efficient positional cloning. Recent development of polymerase chain reaction (PCR)-based markers such as cleaved amplified polymorphic sequence (CAPS)13 or derived-CAPS (dCAPS),14 have made it possible to analyze a large number of plants in a short time in the early growth stage.

By employing advanced positional cloning strategies with high-throughput genetic mapping using CAPS, dCAPS or single nucleotide polymorphisms (SNP) markers, we successfully identified sd-1 as a single open reading frame (ORF). Herein we describe the process of positional cloning of sd-1 which should be useful for producing high-yield cereal crops, and discuss the potential of accelerated positional cloning in plants.

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2. Materials and Methods

2.1. Plant materials

The semidwarf indica cultivar Habataki and the normal-type japonica cultivar Sasanihiki were crossed, backcrossed, self-pollinated, and selected by marker-assisted selection. The selected plant (SBIL5) had close chromosomal similarity to Sasanihiki over the whole genome length with the only heterozygous sequences located on the long arm of chromosome 1, covering the sd-1 locus. A self-pollinated population of SBIL5 consisting of 263 individuals was grown in an experimental paddy field in Tsukuba, Japan, from April to September 2000 for use in the genetic analysis. Self-pollinated seeds of individuals which had sd-1 in a heterozygous chromosomal region were collected and used as the segregating population for a large-scale genetic analysis. In this analysis, sd-1 genotypes of the tested plants were confirmed again by measuring the stem lengths of self-pollinated progeny (progeny testing).

2.2. Positional cloning

To produce markers, primers were designed on the basis of the Nipponbare genome sequence, and the PCR amplification products from Sasanihiki and Habataki were sequenced to search for SNPs, which were converted into CAPS or dCAPS markers. Primers producing PCR products different in fragment length were used as codominant or dominant PCR markers. Primers and restriction enzymes used in PCR, CAPS, and dCAPS analyses are listed in Table 1. For j (SNP marker), an SNP at 81,266-bp of OSJNBa002982 (DDBJ Acc. No.: AC090974), actcctaaC/Tgttgat, was used for genotyping, with a AcycloPrime-FP SNP Detection System (Perkin Elmer Life Science, Boston, MA, USA) and a fluorescence polarization analyzer ARVOsx-8 (Perkin Elmer Life Science).

For cultivation of rice plants, seedling trays with 96 inserts were used to establish a one-to-one correspondence between plants and DNAs, extracted and analyzed in 96-well microtiter plates. DNA was extracted as follows: A 1-cm-long green leaf of a juvenile plant, a zirconium ball 3 mm in diameter (Iuchi-Seieido, Osaka, Japan), and 0.4 mL TPS buffer (100 mM Tris-Cl buffer containing 10 mM EDTA and 1 M KCl) were combined in 1.2-mL microcentrifuge tubes set in a 96-well tube rack (QIAGEN, Venlo, The Netherlands). Then the samples were pulverized twice for 1 min at 30 rps in a Mixer Mill MM300 (QIAGEN). DNA was rescued from the centrifuged supernatant by isopropanol precipitation, dried, dissolved in distilled water, and used as a template for PCR amplification.

2.3. Nucleotide sequence analysis

For determination of expressed sequences, 3′- and 5′-rapid amplification of cDNA ends (RACE)-PCR was performed using a SMART PCR cDNA synthesis kit (Clontech, Palo Alto, CA, USA) with HotStar Taq (QIAGEN). Amplification products were cloned into the pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA, USA) and sequenced with DYEnamic ET terminator reagent (Amersham Biosciences, Tokyo, Japan) in a MegaBACE 1000 DNA Sequencing System (Amersham Biosciences). To avoid PCR-induced errors, we determined DNA sequences from at least four independent clones of each amplified product.

2.4. RT-PCR

Total RNA was extracted by using the single-step guanidium thiocyanate-phenol-chloroform extraction method15 with minor modification, from expanded leaves of Habataki, Sasanihiki, IR24, Calrose, Calrose 76, and Milyang 23 and used for RT-PCR analysis. To investigate transcription levels at different growth stages and in different tissues, total RNA was extracted by the same method from seedlings 24, 48, 72, and 96 hr after sowing; RNA was also extracted from 10-day-old plants, roots of 14-day-old plants, leaves of 30-day-old plants, and flowering panicles of Nipponbare. To amplify the gibberellin 20-oxidase gene (GA20-ox) on the sd-1 locus, RT01 primers (U: 5′-AGCTGGACATGCCGCATTGCCTG-3′ and L: 5′-TTGAGCAGCATTGCCTGCAGGAAG-3′) were used. The positions of these primers are shown by arrows in Fig. 4. To amplify another reported rice GA20-ox, OsGA20-ox (DDBJ Acc. No.: U50333),16 5′-CCTGCCATCGCACAAGCAACT-3′ and 5′-GATGGGTAACGGTGAACCAACA-3′ were designed in the 3′-untranslated region (UTR) of this gene. For controls, primers were designed in the reported rice actin gene sequence (Genbank Acc. No.: X16280): OsActinU (5′-CTGGGTTCGCCGGAGATGAT-3′) and OsActinL (5′-CTGGGTTCGCCGGAGATGAT-3′).

3. Results

The DGWG-type semidwarf indica cultivar Habataki and the normal-type japonica cultivar Sasanihiki (Fig. 1) were used as parents to develop a segregating population, SBIL5. The stem lengths of the SBIL5 progeny (n = 263) varied between 60 and 120 cm at the ripening stage. The ratio of progeny with stem lengths of 60–85 cm to those with stem lengths of 85–120 cm was approximately 1:3 (Fig. 2), indicating that sd-1 can be treated as a mendelian factor in this population. Progeny testing clearly distinguished heterozygous (sd-1/Sd-1) plants. Genetic mapping with the first 263 plants revealed that sd-1 lies between two CAPS markers, E30867 and E61578. These markers were gener-
Table 1. List of PCR-based molecular markers designed in this study.

| Marker | Primer pairs | Fragment Size (bp) | Restriction Enzyme | Polymorphism* |
|--------|--------------|--------------------|--------------------|---------------|
| E3067  | CAPS         | H=287              | Pst I              | H=219, 68     |
|        | dCAPS        | S=283              | Bgl II             | S=283         |
|        | a            | 115                | Bts I              | S=115         |
|        | b            |                    | S: approx. 500     |               |
|        | c            |                    | H<5 (size of product) |               |
|        | d            |                    | H<5 (size of product) |               |
|        | e            |                    | Eco RI             | H=494         |
|        | f            |                    | S=370, 124        |               |
|        | g            | 792                | Msp I              | S=744         |
|        | h            | 774                | Ase I              | S=836         |
|        | i            |                    |                    |               |
|        | j            | 682                |                    |               |
|        | k            |                    |                    |               |

*Polymorphisms for H (Habataki) and S (Sasanishiki). Numbers indicate the fragment size in bp.
b SNP at 81,266-bp of OSJNBa0029802 (DDBJ Acc. No.: AC090974)
c These primers were used for PCR from genomic DNA.
d SNP primer used for AcycloPrime-FP reaction.

Figure 1. Stature of normal-type and semidwarf rice plants at ripening. Right: normal-type (Sasanishiki, a japonica cultivar). Left: semidwarf-type (isogenic line of Sasanishiki with a chromosomal segment containing sd-1 introduced from the semidwarf-type indica cultivar Habataki).

Figure 2. Distribution of stem lengths of each plant in the SBIL5 population at ripening. SBIL5 has close chromosomal similarity to Sasanishiki over the whole genome length and has its only heterozygous sequences on the long arm of chromosome 1, covering the sd-1 locus.
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respectively, from the distal end of the short arm. We then used an additional 978 segregants to narrow down the sd-1 candidate region. Thirty-eight recombinants were obtained between E30867 and E61578. Then we produced four CAPS and three PCR markers (a to g, in Fig. 3a, b) between E30867 and E61578, based on the Nipponbare genome sequence of this region (DDBJ Acc. No.: AC099974) and used them for genotyping these recombinants. With this genetic information and the results of phenotype examination, we mapped sd-1 between two of these markers, d and f (Fig. 3b), separated by 43 kb. Then we performed one more selection of recombinants using 2236 additional segregants. Recombinants obtained between d and f were genotyped with additional markers h (codominant PCR marker), i (CAPS marker), j (SNP marker), and k (codominant PCR marker). Together with the results of progeny testing of these recombinants, sd-1 was localized within a 6-kb interval between h and j (Fig. 3c, d).

According to the results of gene prediction using the “RiceGAAS: Analyses for Your Genomic Sequences” program, which is publicly available on Rice Genome Research Program (RGP) home page (http://rgp.dna.affrc.go.jp/), this 6-kb genomic sequence contains only 1 predictable ORF, that for GA20-ox, which is involved in the biosynthesis of gibberellin (Fig. 3d).

We performed 5′- and 3′-RACE-PCR to determine the nucleotide sequence of the expressed region of the predicted GA20-ox gene, and compared the sequences between IR24, Habataki, Milyang 23 (DGWG-type sd-1 mutants) and Calrose 76 (radiation-induced sd-1 mutant of Calrose), and between Nipponbare, Sasanishiki and Calrose (normal-type cultivars). The result shows that Nipponbare, Sasanishiki, and Calrose have an identical sequences in this region, consisting of 3 exons (558, 318, and 291 bp) and 2 introns (105 and 1471 bp). Among the sd-1 mutants, the DGWG-type mutants (Habataki, Milyang 23, and IR24) have a 383-bp deletion from the genome (278-bp deletion from the expressed sequence), from the middle of exon 1 to upstream of exon 2, including a 105-bp intron, resulting in a frame-shift that produces a termination codon in exon 3 (Fig. 4). Calrose 76 has a 1-bp substitution in exon 2, causing an amino acid substitution (Leu [CTC] to Phe [TTC]).

We analyzed the transcription levels of the GA20-ox gene at the sd-1 locus (GA20ox-sd1) by using RNAs from fully expanded leaves of normal-type cultivars and sd-1 mutants. Accumulation of GA20ox-sd1 transcript was detected in normal-type cultivars (Fig. 5a). Among the DGWG-type sd-1 mutants, IR24 and Habataki had little transcript of this gene, while Milyang 23 expressed a normal or greater amount of truncated transcript. No significant difference was observed between Calrose and its single-nucleotide-substitution mutant Calrose 76.

Analysis of the tissue- and stage-specificity of tran-

Figure 3. a. Genetic map of a semidwarfing gene sd-1 based on a total of 1241 (263 + 978) segregating progeny of SBIL5. S13471, E30867, E61578, S13312, a, b, and c are CAPS or PCR markers located on the long arm of chromosome 1. Numbers below the horizontal bar indicate the number of recombinants detected in each interval. The thick bar indicates the candidate region determined at this stage. b. Detailed genetic and physical relationships between sd-1 and CAPS and PCR markers. d, e, f, and g are CAPS or PCR markers designed on the basis of Nipponbare genome sequence. “Rec” indicates the position of the chromosomal recombination. c. Detailed genetic and physical relationships between sd-1 and CAPS, PCR, and SNPs markers (c-k) as a result of genetic analysis using additional 2236 segregants. d. Graphical genotypes of Habataki, Sasanishiki, and two recombinants (#2529 and #3940) and phenotypes of sd-1 of these plants are summarized. Habataki-type and Sasanishiki-type chromosomes are shown as open and black boxes, respectively. The horizontal line indicates the location of the candidate region of sd-1. e. Predicted genotypes of plants summarized in c. f. Predicted genotypes of plant F5a in c. g. Predicted genotypes of plants summarized in c.
Nucleotide and amino acid sequences of the Milyang 23 and IR24 (DGWG-type shaded box indicates the region that was deleted in Habataki, genes. Letters without amino acid labels represent introns. The substitution in Calrose 76 (“Cal76”) and in Habataki, Milyang and bold letters indicate the position of the single nucleotide.

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gcgccatggtcatcaacatcggcgacaccttcatg
850       860       870
D  V  G  G

gacgtcggcggc
760       770       780
R  T  L  G  T  G  P  H  C  D  P  T  A  L  T  I  L  L  Q  D

cggacgctcggcacgggcccgcactgcgaccccaccgccctcaccatcctcctccaggac
730       740       750       760       770       780
F  A  D  S  S  S  I  M  R  C  N  Y  Y  P  P  C  P  E  P  E

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Figure 4. Nucleotide and amino acid sequences of the Nipponbare, Sasanishiki, and Calrose (normal-type) Sd-1 genes. Letters without amino acid labels represent introns. The shaded box indicates the region that was deleted in Habataki, Milyang 23, and IR24 (DGWG-type Sd-1 mutants). Shaded and bold letters indicate the position of the single nucleotide substitution in Calrose 76 (“Cal76”) and in Habataki, Milyang 23, and IR24 (“DGWG”).

Figure 5. a. Transcription levels of predicted GA20ox-sd1 in normal-type and semidwarf varieties. b. Transcription levels of GA20ox-sd1, OsGA20-ox, and rice actin gene (control) at different growth stages and in different tissues of a normal-type variety, Nipponbare. Total RNA was extracted from seedlings 24, 48, 72, and 96 hr after sowing, 10-day-old plants, roots of 14-day-old plants, leaves of 30-day-old plants, and flowering panicles. For each analysis, 25, 30, and 35 cycles of PCR amplification were tested in advance to determine the cycle numbers in which amplification occurs in the exponential phase. At least three independent amplifications were performed to confirm the reproducibility.

scription of GA20ox-sd1 in Nipponbare revealed that this gene was expressed within 48 hr after sowing, as well as in 10-day-old plants, 30-day-old leaves, and flowering panicles (Fig. 5b); no transcription was detected in 24-hr-old seedlings or in 14-day-old roots. Transcript accumulated predominantly in adult leaves.

4. Discussion

Positional cloning revealed rice semidwarf gene sd-1 encodes a key enzyme in gibberellin biosyn-
thesis, GA20-ox. Expression analysis of this gene (GA20ox-sd1) revealed there are differences in transcription levels among DGWG-type sd-1 mutants. Habataki and IR24 had little transcript of this gene, while Milyang 23 expressed a normal or greater amount of truncated transcript. While the reason for the observed differences in expressed transcript level is unclear, the different genetic background, such as differences in transcription factors or promoter sequences may be responsible. In stage-specific expression analysis of the normal-type cultivar Nipponbare, expression was strongest in adult leaves. This suggests that GA20ox-sd1 contributes to plant height, especially in the leaves of the reproductive phase. Significant expression of another reported GA20-ox gene of rice, OsGA20-ox (DDBJ Acc. No.; U50333), was observed in seedlings (24–96 hr) and flowering panicles, and weak expression was observed in roots, adult leaves and young plants (Fig. 5b). In Arabidopsis, three GA20-ox genes are expressed in different tissues, apparently involving different developmental processes that are controlled by GA. There was no significant difference in the transcription level of the other OsGA20-ox between normal-type cultivars and sd-1 mutants (Fig. 5a). Presumably, the existence and expression of OsGA20-ox prevent severe dwarfishm from developing in sd-1 mutants.

GA20-ox catalyzes the sequential oxidation and elimination of C-20 in the GA biosynthesis pathway, providing substrate for GA3β-hydroxylase (GA3ox), which catalyzes the last step of synthesis of active GAs. It is not surprising that a rice semidwarfing gene encodes GA20-ox since successful production of semidwarf plants using antisense or overexpressed GA20-ox genes has been reported in Arabidopsis, Solanum dulcamara, potato, and lettuce. Although no example in rice has been reported yet, these and our results indicate that rice GA20-ox genes at the sd1 locus, including mutant genes, can be used to increase the yield in existing rice varieties that have low yield but high quality and special commercial value.

In positional cloning, discovery of recombinants in the vicinity of the target gene is the key step; the larger the segregating population scale, the smaller the resultant candidate interval. In Arabidopsis, 400 to 500 segregants narrowed down a candidate interval to 100 to 160 kb, and 2500 to 3000 segregants to < 10 kb. In rice, 1505 segregants gave a 12-kb interval for the photoperiod sensitivity gene Hdl, and 2807 segregants gave a 26-kb interval for Hde. These examples demonstrate that 3000 or more segregants are required to determine a candidate gene as a single ORF by genetic mapping. In this study, we performed genetic mapping three times, with 263, 978, and 2236 segregants (in total 3477), respectively. Determined candidate regions in each analysis were 2.8 cM (corresponds to approximately 560 kb), 43 kb, and 6 kb, respectively. We used 1-week-old rice plants for DNA extraction, and genotyping could be completed within 2 weeks after sowing. This made it possible to cultivate plants in a single tray with 96 inserts, select recombinants and transfer them to larger pots in the early growth stage for trait evaluation. We produced and used 12 PCR-based markers, and one SNP marker, j, was used successfully as a test case. As SNP analysis needs no electrophoresis and no searching or introducing of restriction sites, and SNPs and genome sequencing data have been accumulating rapidly in Arabidopsis and rice, future progress in the development of a cost-effective analytical system of SNPs will definitely accelerate positional cloning in plants.

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