

Semidwarf (*sd-1*), "green revolution" rice, contains a defective gibberellin 20-oxidase gene

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The introduction of semidwarf rice (*Oryza sativa* L.) led to record yield increases throughout Asia in the 1960s. The major semidwarfing allele, *sd-1*, is still extensively used in modern rice cultivars. The phenotype of *sd-1* is consistent with dwarfism that results from a deficiency in gibberellin (GA) plant growth hormones. We propose that the semidwarf (*sd-1*) phenotype is the result of a deficiency of active GAs in the elongating stem arising from a defective 20-oxidase GA biosynthetic enzyme. Sequence data from the rice genome was combined with previous mapping studies to locate a putative GA 20-oxidase gene (*Os20ox2*) at the predicted map location of *sd-1* on chromosome 1. Two independent *sd-1* alleles contained alterations within *Os20ox2*: a deletion of 280 bp within the coding region of *Os20ox2* was predicted to encode a nonfunctional protein in an *indica* type semidwarf (Doongara), whereas a substitution in an amino acid residue (Leu-266) that is highly conserved among dioxygenases could explain loss of function of *Os20ox2* in a *japonica* semidwarf (Calrose76). The quantification of GAs in elongating stems by GC-MS showed that the initial substrate of GA 20-oxidase activity (GA_{53}) accumulated, whereas the content of the major product (GA_{20}) and of bioactive GA_1 was lower in semidwarf compared with tall lines. We propose that the *Os20ox2* gene corresponds to the *sd-1* locus.

The semidwarfing gene in rice (*sd-1*) is one of the most important genes deployed in modern rice breeding. Its recessive character results in a shortened culm with improved lodging resistance and a greater harvest index, allowing for the increased use of nitrogen fertilizers (1, 2). The *sd-1* gene was first identified in the Chinese variety Dee-geo-woo-gen (DGWG), and was crossed in the early 1960s with Peta (tall) to develop the semidwarf cultivar IR8 (3), which produced record yields throughout Asia and formed the basis for the development of new high-yielding, semidwarf plant types (3). Since the 1960s, *sd-1* has remained the predominant semidwarfing gene present in current rice cultivars.

Many aspects of plant growth and development involve the gibberellin (GA) hormone family. The widely used semidwarfing genes in wheat, *Rht-B1b* and *Rht-D1b*, encode a protein involved in GA-signal transduction (4). In addition, GA-responsive dwarf mutants have been identified in many species, often resulting from mutations in genes encoding GA biosynthetic enzymes (reviewed in ref. 5). In rice, mutants *dx* and *d18* are defective in *ent*-kaurene oxidase and GA 3-oxidase activity, respectively, representing early and late steps in the GA biosynthetic pathway (6, 7). These dwarf plants have a low content of GA_1 (a major growth active GA in rice) and can be rescued by application of active GAs such as GA_3 . Previous studies (8, 9) have suggested that the *sd-1* semidwarf results from a deficiency in active GAs, although the methods used (enzyme-linked immunosorbent or bioassay) lack the precision of modern chemical methods of analysis. Furthermore, *sd-1* plants retained responsiveness to GA_3 during seedling, tillering, and heading growth stages (8, 10). These properties of *sd-1* are consistent with a semidwarf phenotype that is the result of a partial block in GA biosynthesis. We have identified a putative GA 20-oxidase gene very close to the *Sd-1* locus on chromosome 1. We show that semidwarf mutants contain alterations in this gene, leading to changes in GA content that are consistent with a block at this step

in the GA biosynthetic pathway and, therefore, propose that the GA 20-oxidase gene corresponds to the *Sd-1* locus.

Materials and Methods

Plant Material. Kyeema is a tall *indica* type rice cultivar developed in the Australian breeding program at Yanco, Australia. Its pedigree includes Pelde and Kulu (Yanco varieties) and Della, a tall long grain (fragrant) variety from Louisiana (USA). The semidwarf, *indica* type Doongara was derived from a cross between a tall, long grain cultivar Dawn (Texas) and IR8, a semidwarf cultivar carrying *sd-1* which was developed at the International Rice Research Institute (IRRI) in the 1960s (3). IR36 was developed from IR8 at IRRI and also carries *sd-1*. The dwarfing gene in short-stature *japonica* type Calrose76 (a mutant isolated in the tall Californian cultivar Calrose following irradiation) was shown to be allelic to *sd-1* (11).

Analysis of GA Content. Kyeema and Doongara plants were grown at 29°C day/22°C night temperatures during the summer season in a phytotron glasshouse. Stem material was harvested when the top internode of the stem had reached approximately 50% of final length, with average lengths of 139 mm in Kyeema, 101 mm in Doongara, 134 mm in Calrose, and 117 mm in Calrose76. The tissue was frozen in liquid N₂ and stored at -80°C. Frozen stem segments were ground in liquid N₂ with sand in a mortar and transferred to a beaker with 80% methanol. Extraction, purification, and analysis of GAs and abscisic acid (ABA) by GC-MS using selected ion monitoring was by methods that have been described (12).

PCR Amplification of Genomic DNA. PCR primers for the putative GA 20-oxidase gene were designed on the basis of the genomic sequence of Nipponbare available on the Rice Genome Research Project (RGP) database (<http://rgp.dna.affrc.go.jp>). The predicted ORF was amplified from Kyeema with the following primers: forward primer, 5'-CAACTCACTCCCCTCAACACAGC, and reverse primer, 5'-TTTCAAATGCAATGTCGTCCACC were used to amplify exon 1; forward primer, 5'-GCGCCAATGGGG-TAATTAACAACG and reverse primer, 5'-GGCATTCCATT-GTTTGTGATTGG were used for exon 2; forward primer, 5'-GTTTGTCTTGTGCGGTTGCTCAG and reverse primer, 5'-TCTGTTTCGTTCCGTTTCGTTCCG were used for exon 3. The 5' region in Doongara containing a deletion in exon 1 and exon 2 (see *Results*) was amplified by using the forward primer for exon 1 and the reverse primer for exon 2. The PCR analysis (20 μl) contained 100 ng of template DNA, 1 × PCR buffer (Qiagen, Germany), 1.5 mM MgCl₂, 0.2 mM dNTPs, 5 μM primer, and 1 unit of *Taq* polymerase (Hotstar, Qiagen). The reactions were heated to 94°C for 5 min followed by 35 cycles of amplification at 94°C for 30 sec, 55°C for 30 sec, and 72°C for 50 sec. PCR products were cloned into a pGEM-T Easy Vector (Promega), and at least two indepen-

Abbreviation: GA, gibberellin; RGP, Rice Genome Research Project.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AY114310).

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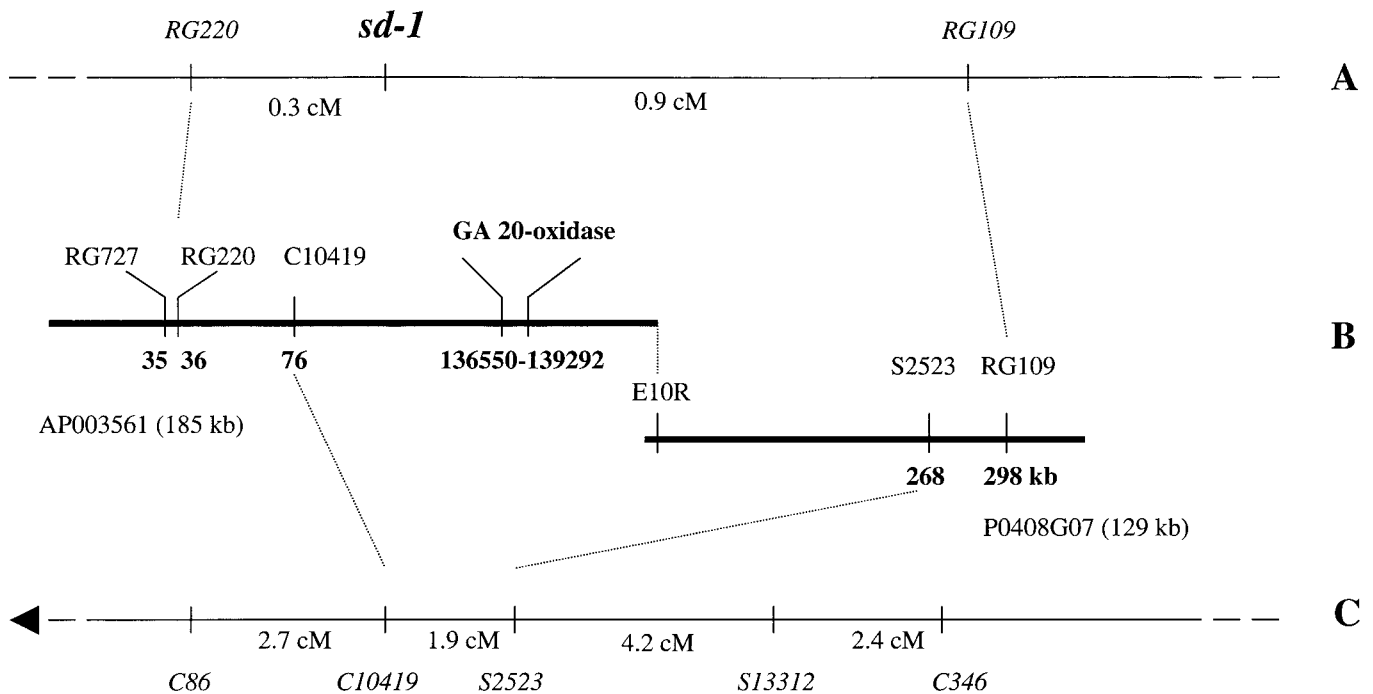


Fig. 1. Genetic linkage maps and BAC contig of the *sd-1* region on chromosome 1 of rice. (A) Genetic linkage map of *sd-1* region (16). (B) Physical contig of DNA sequence flanking *sd-1* consisting of BAC clones AP003561 and P0408G07 (RGP). (C) High-resolution genetic linkage map of the *sd-1* region on chromosome 1 (RGP 2000). Arrow indicates the direction of the centromere.

dent clones from each PCR product were sequenced by using an automated sequencing system (ABI 377, Applied Biosystems).

RT-PCR Analysis of OsGA20ox2 Transcripts. To isolate the *GA20ox2* cDNA, RNA was extracted according to Vries *et al.* (13) from elongating stems of Calrose and Calrose76 rice plants. The stem material comprised the upper internode and flag leaf sheath and was harvested at the stage where the panicle emerged from the flag leaf sheath. The cDNA was amplified by using a one-step RT-PCR kit (Qiagen) according to the manufacturer's protocols, with a 58°C annealing temperature and forward primer 5'-CAACTCACTC-CCGCTCAACACAGC-3' located 22-bp upstream from the translation start, and a reverse primer 5'-GTTCGTTCCGTTTCGGT-TCCG-3' eight-bp downstream from the TGA stop codon. The RT-PCR products were gel-purified and ligated into pGEM-T-Easy vector (Promega).

DNA Blot Analysis. Genomic DNA was extracted by using the methods described by Dellaporta *et al.* (14). DNA probe from exon 2 of *Os20ox2* was amplified from genomic DNA by using primer sequences described above. DNA hybridization was carried out with 10 µg of *Hind*III-digested genomic DNA by using methods described by Lagudah *et al.* (15). Filters were washed twice in 2× SSC, 0.1% SDS at 65°C for 20 min, once in 1× SSC, 0.1% SDS at 65°C for 20 min, and once in 0.5× SSC, 0.1% SDS at 65°C for 15 min.

Results

Genetic and Physical Analysis of the *Sd-1* Locus. The rice *sd-1* dwarfing gene was shown to be tightly linked to restriction fragment length polymorphism (RFLP) markers *RG109* and *RG220* on the long arm of chromosome 1 in at least three mapping studies. First, in 178 F₂ segregants from a cross between Shiohari and a near-isogenic semidwarf line (ID47), the *sd-1* gene was flanked by *RG109* on the proximal (0.9 cM) and *RG220* on the distal side (0.3 cM; ref. 16). Second, the analysis of 2,450 segregating lines derived from a cross between near-isogenic lines Taichung65 and

Taichung65 (*sd-1*) separated *sd-1* from *RG109* by 0.2 cM (17). Third, in 50 F₂ segregants from a cross between Kyeema and Doongara (*sd-1*), *RG109* co-segregated with *sd-1*, whereas one recombinant was identified between *RG220* and *sd-1* (≈1 cM) (S. Garland, personal communication). In this study, we positioned RFLP markers *RG109* and *RG220* on a physical segment of chromosome 1 covered by a BAC contig of approximately 300 kb by using the physical map of Nipponbare available on the RGP database (<http://rgp.dna.affrc.go.jp>; Fig. 1). These markers were positioned near the ends of this contig spanning a physical distance of approximately 262 kb. Anchor markers *C10419* and *S2523*, located within this region and separated by 192 kb, were mapped 1.9 cM apart on the high-resolution linkage map (RGP 2000). From this chromosomal segment and linkage map, we estimated that the ratio of physical to genetic distance was approximately 100 kb/cM in this region. When combined with data from the previous mapping studies, these results indicated that the *sd-1* gene was located within a 262-kb interval flanked by markers *RG109* and *RG220*.

Identification of a Putative GA 20-Oxidase in the *sd-1* Region. Because the *sd-1* phenotype is consistent with the dwarfism that results from a deficiency in bioactive GA₁, we investigated whether GA biosynthetic genes were present near the *Sd-1* locus. The Rice Genome Automated Annotation system (RiceGAAS, RGP) was used to identify predicted coding regions within the interval flanked by RFLP markers *RG109* and *RG220*, as well as within 500 kb of sequence on either side of these markers. Most of the 28 predicted coding regions located between *RG109* and *RG220* were related to hypothetical proteins of unknown function or retrotransposable elements. However, one predicted ORF (between positions 136550–139292 on BAC clone AP003561, Fig. 1) encoded a protein of 389 amino acids that was closely related to GA 20-oxidases previously isolated from *Arabidopsis* (*GA5*, 47% identity), pea (50% identity), rice (51% identity; refs. 18–20), and related GA 20-oxidase sequences from cereals such as wheat (Fig. 2). There was

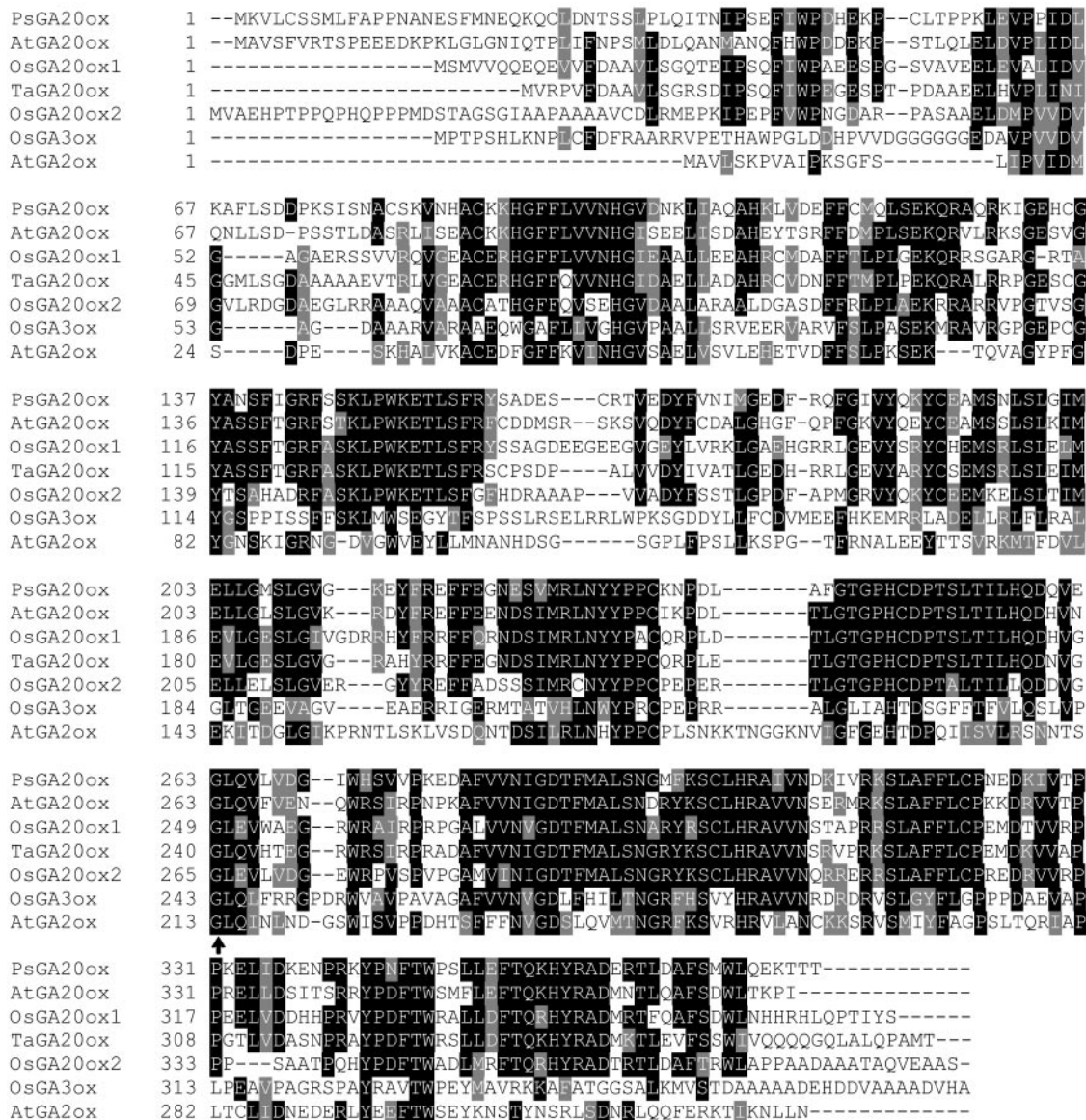


Fig. 2. Alignment of predicted dioxygenase proteins. Dark shading indicates amino acid residues that are conserved in the GA 20-oxidases. GenBank accession numbers are: pea, *PsGA20ox*, PSU58830; *Arabidopsis*, *AtGA20ox*, AAC39314; wheat, *TaGA20ox*, TAY14007; rice, *OsGA20ox1*, U50333; *OsGA20ox2*, AY114310; *OsGA3ox*, AB056519; *AtGA20ox*, AJ132435. Arrow indicates the highly conserved leucine residue.

no other predicted coding region identified that contained significant amino acid sequence relatedness to proteins involved in GA biosynthesis in the regions either proximal or distal to the BAC contig. Genetic analysis has linked a mutant GA 20-oxidase gene to a semidwarf phenotype in *Arabidopsis* (18). Therefore, we investigated the possibility that the *sd-1* allele is associated with a mutation in the putative GA 20-oxidase gene.

Characterization of Mutant GA 20-Oxidase Genes in *sd-1* Lines. The putative GA 20-oxidase gene (*Os20ox2*) was amplified by PCR by using genomic DNA of cvs Kyeema (tall) and Doongara (*sd-1*) as template and primers developed from the Nipponbare genomic sequence (<http://rgp.dna.affrc.go.jp>). Sequence analysis of these PCR products demonstrated that the predicted ORF isolated from Kyeema was identical in sequence to Nipponbare. Intron positions (557–558 and 879–880 in Fig. 3) were confirmed by RT-PCR (see *Materials and Methods*) and were conserved in relation to GA 20-oxidase genes isolated from *Arabidopsis* and pea (18, 19). The

corresponding sequence amplified from the semidwarf cultivar Doongara contained a 280-bp deletion within the coding region. This deletion spanned parts of exon 1 and exon 2 and was predicted to result in a truncated, nonfunctional polypeptide containing the first 99 amino acid residues of the GA 20-oxidase, followed by an additional 3 amino acids that were in the wrong reading frame before a termination codon (Fig. 3). Hybridization of a DNA probe derived from exon 2 to digested genomic DNA of Kyeema (tall), Doongara (*sd-1*), and IR36 (*sd-1*) resulted in an RFLP pattern which was consistent with a deletion in the putative GA 20-oxidase gene (Fig. 4). The RFLP analysis identified two gene members in the rice genome: a strong band (4.6 kb) of the predicted size corresponding to a single member at the *sd-1* locus, and a weaker band (\approx 8 kb) probably identifying a related GA 20-oxidase gene (*Os20ox1*) located on chromosome 3 (GenBank U50333; ref. 20).

We investigated whether an independent semidwarf mutant, allelic to *sd-1*, also contained an altered *Os20ox2* sequence by

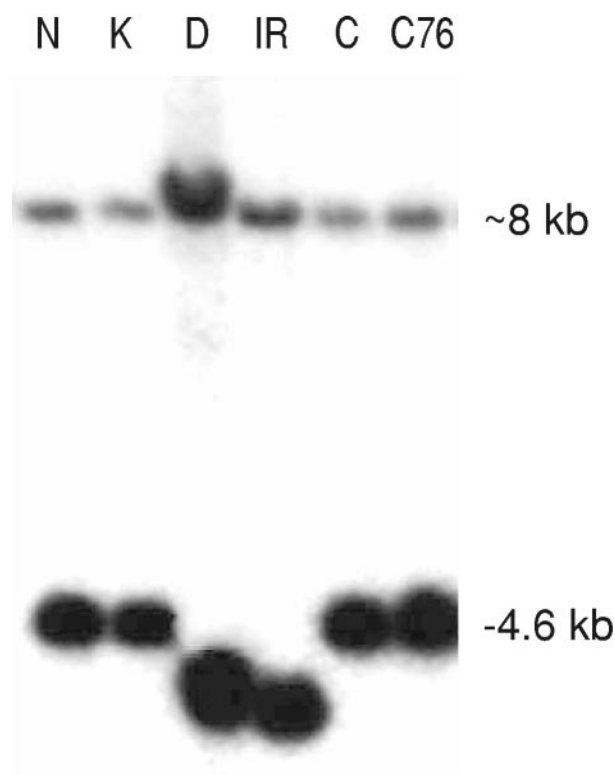


Fig. 4. Hybridization of DNA probe of exon 2 derived from the putative GA 20-oxidase gene (*Os20ox2*) to (*Hind*III) digested genomic DNA of Nipponbare (N, tall), Kyeema (K, tall), Doongara (D, *sd-1*), IR36 (IR, *sd-1*), Calrose (C, tall), and Calrose76 (C76, *sd-1* allele).

Analysis of GA Contents in Elongating Stems. Most of the variation in final plant height between tall and semidwarf lines result from differences in stem length of the first (subtending panicle) and second (subtending flag leaf) stem internodes (24). We initially compared the GA contents of Kyeema (tall) and Doongara (*sd-1*)

by using the uppermost stem internodes at equivalent stages of growth (about 50% of final length). The results (Table 1) showed a higher (about three-fold) content of GA₅₃ in the semidwarf compared with the tall line, and lower (about two-fold) contents of GA₂₀ and GA₁, and possibly GA₁₇, which is derived from GA₁₉ (Table 1). There was no significant difference between semidwarf and tall lines in the contents of GA₄₄ and GA₁₉. This analysis also revealed that early 13-hydroxylated GAs predominated in the stem segments, and the peaks identified for GA₉ and GA₃₄ were just above the detection limits. The elevated content of GA₅₃ in the semidwarf, and the reduced amount of GA₂₀ (and GA₁) were consistent with impaired GA 20-oxidase activity (Fig. 5) and dwarfing caused by a deficiency of GA₁. Because *sd-1* rice still contained considerable amounts of GA₄₄, GA₁₉, GA₂₀, and GA₁, yet is proposed to have a nonfunctional *Os20ox2* gene, we conclude that there must be another functional GA 20-oxidase isozyme whose activity provides biosynthetic precursors to GA₁ in the stem segment.

We also compared GA contents in equivalent stem segments of Calrose and Calrose76. The results (Table 1) were similar to those above, in that there was an accumulation of GA₅₃ and a deficiency of GA₂₀ in the semidwarf variety, consistent with reduced activity of a GA 20 oxidase. The extent of these changes was slightly less than that observed for the Doongara/Kyeema pair, as was the degree of reduction in GA₁ content of Calrose76 compared with Calrose (nonsignificant at $P < 0.05$). Calrose76 also had lower contents of GA₄₄ and of GA₁₉ than Calrose. These differences might be because of (i) other genetic differences between the Doongara/Kyeema pair, (ii) slight activity of the mutant GA20ox2 gene product in Calrose76, or (iii) experimental variation.

Discussion

The data from the public rice genome sequence was combined with previous mapping studies to locate a putative GA 20-oxidase gene (*Os20ox2*) at the expected position of *sd-1* on chromosome 1. *Os20ox2* was the only predicted ORF identified within a target interval of 262 kb that contained significant amino acid sequence relatedness to proteins involved in GA biosynthesis. Two independent semidwarf, allelic mutants contained alterations within *Os20ox2*: a deletion within *Os20ox2* was predicted to encode a nonfunctional protein in Doongara

Table 1. Contents of GAs in equivalent stem segments of tall vs. semidwarf rice plants

Compound	Content (pg segment ⁻¹)*					
	Kyeema (tall)	Doongara (dwarf)	<i>P</i> [†]	Calrose (tall)	Calrose 76 (dwarf)	<i>P</i> [†]
13-OH GAs[‡]						
GA ₅₃	802 ± 54	2546 ± 133	<0.001	1117 ± 72	2033 ± 146	0.005
GA ₄₄	687 ± 34	661 ± 42	0.65 (ns)	828 ± 109	445 ± 61	0.04
GA ₁₉	4092 ± 269	4108 ± 401	0.97 (ns)	4233 ± 141	2827 ± 171	0.003
GA ₂₀	178 ± 13	73 ± 4	<0.001	270 ± 27	131 ± 18	0.012
GA ₁	146 ± 8	96 ± 6	0.005	169 ± 15	134 ± 13	0.15 (ns)
GA ₈	<dl [§]	<dl [§]		nd	nd	
GA ₁₇	395 ± 13	109 ± 20	<0.001	nd	nd	
Non-13-OH GAs[¶]						
GA ₉	7.9	6.1		nd	nd	
GA ₄	interference	interference		nd	nd	
GA ₃₄	7.1	5.7		nd	nd	

*Contents estimated using calibration curves for all GAs except for GA₁₇ (estimated from the peak area of ion 492 relative to the corrected peak area of ion 436 of deuterated GA₁₉) and GA₅₃ (estimated on the basis of corrected 448/450 peak areas). Values are mean ± SE of three (Doongara, Calrose, Calrose 76) or four (Kyeema) independent samples, except for GA₈, GA₉, and GA₃₄, which are single determinations.

[†]Probability (*t* test) that the difference between the means is due to chance.

[‡]GAs are shown in metabolic order except for GA₁₇, a tricarboxylic acid, which represents a branch off the biosynthetic pathway from GA₁₉. GA₁ is the only bioactive GA in this sequence.

[§]Endogenous GA₈ was below the detection limit.

[¶]GA₉, GA₄, and GA₃₄ are equivalent to GA₂₀, GA₁, and GA₈ in the 13-OH GAs. GA₄ was not determined because interfering ions were present.

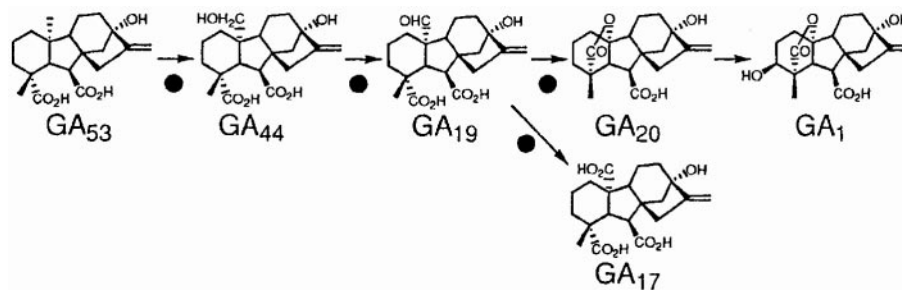


Fig. 5. The early 13-hydroxylation pathway of gibberellin biosynthesis in higher plants. Black circles indicate oxidation steps catalyzed by GA 20-oxidase (adapted from ref. 20).

(DGWG source of *sd-1*), whereas a change in an amino acid residue (Leu-266), which was highly conserved among dioxygenase sequences, could explain the loss of function of Os20ox2 in the semidwarf mutant Calrose76. The quantification of GAs in elongating stems revealed that the initial substrate of GA 20-oxidase (GA₅₃) accumulated, whereas the content of the major product (GA₂₀) was reduced in semidwarf compared with tall lines. These results were consistent with impaired GA 20-oxidase activity and dwarfing caused by a deficiency of GA₁. Therefore, we propose that the semidwarf (*sd-1*) phenotype of rice is the result of reduced GA 20-oxidase activity, and that the defective GA 20-oxidase gene defines the *sd-1* locus.

The determination of GA contents in elongating stems revealed very high levels of GA₁₉, as observed previously in rice seedlings (20). In both semidwarfs, there was a lowered content of GA₂₀, presumably a consequence of reduced 20-oxidase activity. In turn, the amounts of GA₁, a major growth active GA, were only 65% (Doongara/Kyeema) or 80% (Calrose76/Calrose) of the corresponding tall values, although in the latter case the difference was not significant. This modest reduction in GA₁ content is consistent with the relatively small reduction (approximately 25%) in the height of semidwarf vs. tall lines. The mutant GA 20-oxidase in Doongara should be a nonfunctional polypeptide, so our results imply that rice contains additional 20-oxidase activity that provided precursors contributing to approximately half of the GA₁ content found in the stem internodes. A GA 20-oxidase mapping to chromosome 3 could account for the GA₂₀ detected in stems (20). Similarly in *Arabidopsis*, the GA-responsive semidwarf mutant *ga5* resulted from a defective GA 20-oxidase gene, and other gene

members were predicted to supplement GA 20-oxidase activity resulting in a semidwarf phenotype (18).

The *sd-1* gene in rice and *Rht* genes in wheat have played similar roles in height reduction associated with significant yield increases. The molecular bases for semidwarf stature seem to be different, with *sd-1* resulting from a mutant GA 20-oxidase gene causing a deficiency of bioactive GA in elongating stems, whereas *Rht* genes are negative regulators of GA signaling that in the mutant are no longer modulated by GA (4). Unlike *Rht* semidwarf wheat, *sd-1* semidwarf rice retains the ability to respond to applications of bioactive gibberellins. Most of the dwarf mutants identified in rice (*d1* to *d60*), including those resulting from mutations in GA biosynthetic genes (*dx* and *d18*), are not used in crop improvement because they are associated with severe dwarfism, floret sterility, or abnormal plant and grain development (25). The agronomic success of *sd-1* may be intimately linked to the developmental timing of its moderate dwarfing in relation to stem growth and panicle development. It will be of considerable interest to determine the detailed expression patterns of the two 20-oxidase isozymes in different parts of the rice plant, because this determination will underlie physiological interpretations of semidwarf productivity.

During the final stages of the review process of this manuscript, Monna *et al.* (26) and Sasaki *et al.* (27) described independent mutations in the GA20-oxidase gene located at the *sd-1* locus, two of which confirmed the sequence alterations reported here.

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