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Research Communication

Loss-of-function of a Rice Gibberellin Biosynthetic Gene, *GA20 oxidase* (*GA20ox-2*), Led to the Rice 'Green Revolution'

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A rice semi-dwarf variety, IR8, known as "miracle rice" enabled dramatic increases rice production and its widespread adoption averted predicted food shortages in Asia during the 1960s to 1990s. This remarkable achievement was referred to as "green revolution". The short stature of IR8 was derived from the semi-dwarf gene, *sd1*, and the *sd1* gene contributed significantly to the rice "green revolution". In this paper, we described the physiological, molecular genetic and biochemical characterization of the *sd1* gene. The *sd1* mutant contained lower gibberellin (GA) levels than wild-type plants but responded sensitively to exogenous GA. Cloning and sequence analyses revealed that the *SD1* gene encoded a GA biosynthetic enzyme, GA20 oxidase. In all of the *sd1* mutants tested, nucleotide deletions or substitutions were observed in the GA20 oxidase gene (*GA20ox-2*), which induced an internal stop codon or single amino acid substitutions, respectively. The *sd1* plants, which the wild-type *GA20ox-2* gene was introduced showed the normal height. A recombinant GA20ox-2 protein produced from the cDNA clone in *E. coli* catalyzed the conversion of GA_{53} to GA_{20}. These results confirmed that *SD1* encodes an active GA20 oxidase. The expression of *GA20ox-2* was down-regulated by GA in a similar manner to that of some GA20oxs in other plants. The rice genome carried at least two GA 20-oxidase genes (*GA20ox-1* and *GA20ox-2*) and *SD1* corresponded to *GA20ox-2*, which is highly expressed in the leaves and flowers, whereas *GA20ox-1* is preferentially expressed in the flowers. The reduced plant height associated with the *sd1* alleles was due to the low amount of active GA in leaves, which was caused by a mutation of the *GA20ox-2* gene. On the basis of these results, we discussed the importance of GA in the regulation of plant height in crop breeding.

Key Words: rice, green revolution, gibberellin, *sd1*, *Rht.*

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Introduction

Rice (*Oryza sativa* L.) is one of the most important staple foods and it has been estimated that 50% of the human population depends on it as main source of nutrition (White 1994). It is particularly important for people living in the monsoon areas of Asia where it has a long history of cultivation.

In the 1960s, the rapid expansion of the world population and dramatic decrease in cultivated lands raised concerns that food production would not meet the growing demand, leading to a global food crisis. Recognizing the problem, the International Rice Research Institute (IRRI) developed a high-yielding semi-dwarf variety of rice, IR8, known as 'miracle rice'. Widespread adoption of IR8 led to major increases in rice production and, as a result, the feared food shortages were averted in Asia. This remarkable achievement was referred to as 'green revolution' (Hargrove and Cabanilla 1979, Dalrymple 1986, Khush 1999). The short stature of IR8 (Fig. 1-2) was due to the *sd1* mutation, which played an important role for rice growth.

Generally speaking, nitrogen fertilization is essential to increase grain production, but it also induces culm elongation, resulting in an overall increase in the height of crop plants. Tall crop plants are easily flattened by wind and rain and consequently dramatic yield losses may occur. IR8 was bred by the crossing with a Taiwanese native semi-dwarf variety, Dee-geo-woo-gen, which carries the semi-dwarf *1* (*sd1*) gene, and an Indonesian good-taste variety, Peta (Hargrove and Cabanilla 1979, Dalrymple 1986). The resultant IR8 variety showed the semi-dwarf phenotype caused by

the *sd1* allele derived from Dee-geo-woo-gen. The IR8 successfully resolved the lodging problem, because it responded to fertilizer inputs to produce an increased yield without culm elongation.

Like IR8, the high-yielding varieties Taichung Native 1 in Taiwan (Aquino and Jennings 1966) and Tongil in Korea (Suh and Heu 1978) also harbored the *sd1* allele from Dee-geo-woo-gen, and contributed to food security in these countries. Similarly, the native semi-dwarf rice variety Jikkoku (Kikuchi *et al.* 1985), γ-ray induced variety Reimei in Japan (Futsuhara *et al.* 1967), and the γ-ray induced variety Calrose76 in the United States of America (Foster 1978) carried different *sd1* alleles and were widely used in the rice breeding programs in these countries (Fig. 1). The fact that such different *sd1* alleles have been used in rice breeding programs for both indica and japonica subspecies demonstrates the suitability of the *sd1* locus for controlling the height of rice plants (Hargrove *et al.* 1980). The *sd1* mutants have been analyzed using various approaches and these studies have been applied in rice breeding programs (Futsuhara *et al.* 1967, Suge 1975, Kikuchi *et al.* 1985).

In this report, we describe the physiological, molecular genetic and biochemical characterization of the *SD1* gene. The isolation and characterization of the rice *SD1* gene is not only important for elucidating its historical significance, but also provides novel strategies for use in future rice breeding programs.

Materials and Methods

Plant materials

Figure 1 shows the *sd1* mutants that we used in our experiments. Three *sd1* mutants and their corresponding wild-type cultivars were compared, that is, Dee-geo-woo-gen (*sd1*) and Woo-gen (wild-type), Calrose76 (*sd1*) and Calrose (wild-type), and Reimei (*sd1*) and Fujiminori (wild-type). Since Jikkoku is a native variety (japonica), its corresponding wild-type strain has not been identified and therefore we used Taichung 65 (japonica) as a wild-type comparison for Jikkoku in this study. IR8 (*sd1*) was also used in the sequence analysis for *sd1*. Seed materials, Dee-geo-woo-gen, Woo-gen, Calrose76 and Calrose, were kindly provided by the National Institute of Agrobiological Sciences and Aichi Prefectural Agricultural Research Center. Seed materials, Jikkoku, Reimei and Fujiminori were stored at Nagoya University.

Analysis of gibberellin (GA) response and GA content

Ten rice seeds were placed on agar containing various concentrations of GA_3 and incubated at 30°C under continuous light. After 5 days of incubation, the length of the second leaf sheath was measured. Quantitative analyses of the endogenous GAs were performed by gas chromatography-selected ion monitoring (Kobayashi *et al.* 1995).

Molecular cloning, mapping and DNA sequencing

Degenerate primers were designed from the consensus sequences of the *GA20* oxidase gene (*GA20ox*) (Toyomasu *et al.* 1997) from rice and *Arabidopsis* (*GA5*) (Xu *et al.* 1995) (sense: 5′YTNCCNTGGAAYGARACNYT3′ and antisense: 5′GTNGGRTCRCARTGNGG3′). The DNA fragment was amplified by PCR with the genomic DNA from a japonica rice variety, TC65. Using the amplified DNA as a probe, genomic DNA and cDNA clones were identified from the genomic and cDNA libraries. The genomic and cDNA clones from the wild-type and *sd1* alleles were sequenced. To map the *GA20ox-2* gene on the rice genome, a linkage analysis was performed using a population of backcrossed inbred lines derived from the cross between Nipponbare (japonica) and Kasalath (indica) (Lin *et al.* 1998). The linkage was calculated using the MAPMAKER program (Lander *et al.* 1987). Accession number of *GA20ox-2* is AB077025.

Complementation analysis

The genomic *GA20ox-2* sequence (~10 kb) from the wild-type (TC65), including the entire coding region and the 5′ and 3′ flanking regions, was inserted into the binary vector pBI101-Hm2 (Sato *et al.* 1998). The construct was introduced into the Jikkoku *sd1* allele by an *Agrobacterium*-mediated transformation (Hiei *et al.* 1994).

In vitro functional assay

The full-length cDNA was excised and inserted in the sense orientation as a translational fusion product into the pMAL-c2 expression vector and expressed in the *E. coli*, strain JM109. The GA-catalyzing activity of the recombinant protein was measured by full-scan GC-MS (Kobayashi *et al.* 1996).

Expression analysis

Total RNAs were extracted from leaves and flowers. RT-Southern blot analysis was performed using the 1st strand cDNA and the amplified products were separated on an agarose gel and transferred to Hybond N^+ membrane. The DNA gel blot analysis was done by using the coding sequences of *GA20ox-1* and *GA20ox-2* as probes. For the analysis of feedback repression, total RNAs were extracted from the seedlings treated with $\pm 10^{-4}$ M GA_3 or $\pm 10^{-6}$ M uniconazole (an inhibitor of GA biosynthesis) and the DNA gel blot analysis was performed with the *GA20ox-2* sequence as a probe. Actin was used as a control.

Results

Physiological characterization of sd1

The dwarf phenotype in plants can be attributed to various causes, but one of the most important factors for determining plant height is the presence of gibberellins (GAs) known as one of the plant hormones. GAs consist of a large family of tetracyclic diterpenoids and are associated with a

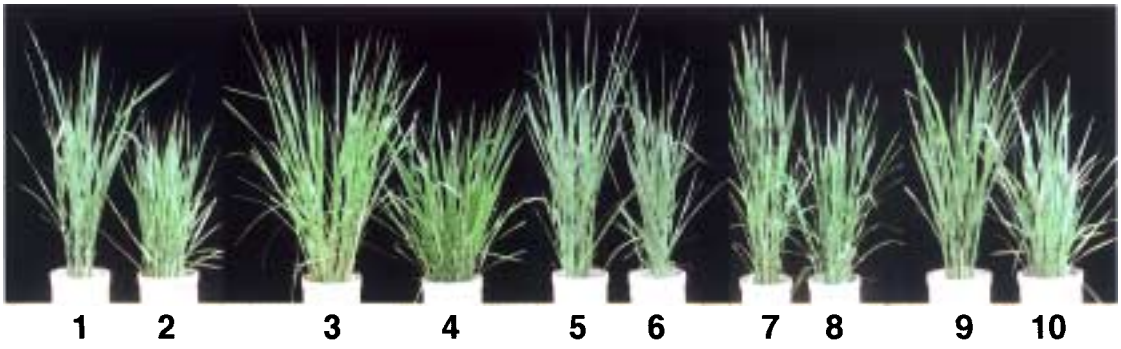

Fig. 1. Morphology of the *sd1* mutant rice plants. 1, Taichung 65 (wild-type); 2, IR8 (*sd1*); 3, Woo-gen (wild-type); 4, Dee-geo-woo-gen (*sd1*); 5, Calrose (wild-type); 6, Calrose76 (*sd1*); 7, Fujiminori (wild-type), 8; Reimei (*sd1*); 9, Taichung 65 (wild-type), 10; Jikkoku (*sd1*).

number of plant growth and developmental processes such as seed germination, stem elongation, flowering and fruit development (Reid 1993, Hooley 1994, Ross *et al.* 1997). To date, we have screened and characterized several rice GA-related mutants and isolated the genes involved in GA biosynthesis and signal transduction in the rice plant (Ashikari *et al.* 1999, Ueguchi-Tanaka *et al.* 2000, Ikeda *et al.* 2001, Itoh *et al.* 2001, Itoh *et al.* 2002). Through these studies, we recognized that the plant morphology of the *sd1* mutants was similar to that of GA-deficient mutants with a weak phenotype.

To determine whether the *SD1* gene might be related to GA, we examined the GA response in the *sd1* mutant. As a first step, we compared the elongation of the second leaf sheath in an *sd1* mutant (Dee-geo-woo-gen) and wild-type (Woo-gen) in response to various exogenous GA_3 treatments. The *sd1* seedlings responded better than the wild-type and the sheath length of *sd1* recovered, becoming similar to that of the wild-type at 10^{-6} M of GA_3 (Fig. 2a). This result suggests that *sd1* may be a GA-deficient mutant.

Genetic analysis of sd1

The steps in the GA biosynthetic pathway in plants have been described previously: bioactive GA_1 and GA_4 are catalyzed from geranylgeranyl diphosphate (GGDP) by the sequential action of cyclases in the plastids, membrane-associated mono-oxygenases in the endoplasmic reticulum, and soluble 2-oxoglutarate-dependent dioxygenases (2ODD) in the cytosol (Hedden and Phillips 2000) (Fig. 2b). In *Arabidopsis*, six enzymes (CPS, KS, EKO, EKAO, GA20ox and GA3ox) have been identified as GA-biosynthetic enzymes (Hedden and Phillips 2000). Recent progress in the rice genome project (Sasaki 1998) has enabled to readily identify rice genes that are homologous to valuable genes that have been isolated in other plant species, such as *Arabidopsis* and maize. Using this information, we have

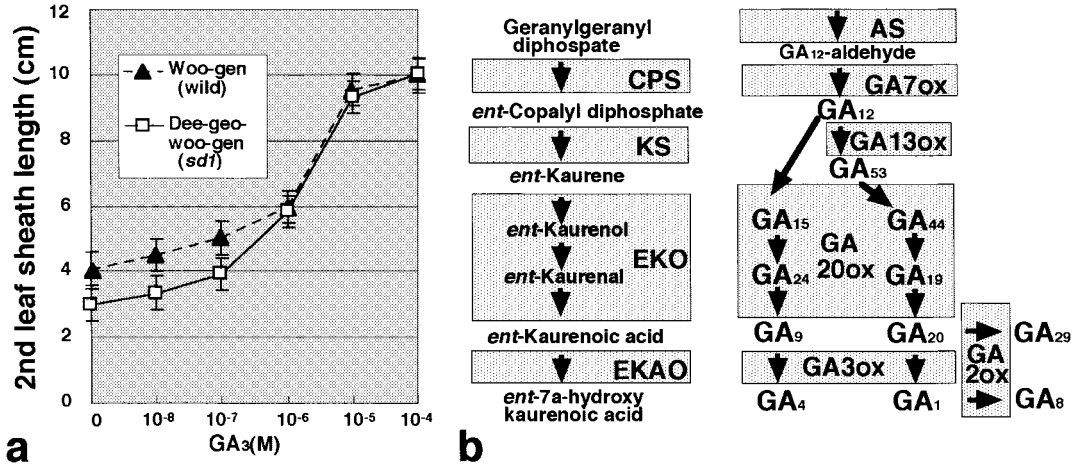

Fig. 2. Response of the *sd1* mutant to GA and schematic GA-biosynthetic pathway.
a. Effect of exogenous GA treatment on elongation of the second leaf sheath. Dee-geo-woo-gen, *sd1* (□), and Woo-gen, wild-type (▲).
b. Schematic representation of the GA-biosynthetic pathway in higher plants. CPS, *ent*-copalyl pyrophosphate synthase; KS, *ent*-kaurene synthase; EKO, *ent*-kaurene 19-oxidase; EKAO, *ent*-kaurenoic acid 7β-hydroxylase; AS, GA_{12}-aldehyde synthase; GA7ox, GA 7-oxidase; GA13ox, GA 13-hydroxylase; GA20ox, GA 20-oxidase; GA3ox, GA3β-hydroxylase; GA2ox, GA 2-oxidase.

identified 5 GA-biosynthetic enzyme-genes from rice (CPS, KS, EKO, EKAO and GA3ox). A mapping analysis of these genomic clones on the rice chromosome revealed that not all of these genes corresponded to the *sd1* gene. Our research team and Hirochika's group in the Rice Genome Research Program in Tsukuba have also isolated knockout mutants for these GA-biosynthetic enzymes, all of which showed a much stronger dwarf phenotype than *sd1* (unpublished data), supporting the assumption that the *SD1* gene does not encode these enzymes, but encodes the remaining enzyme in the GA-biosynthetic pathway.

Analysis of GA contents in sd1

To identify which enzyme is encoded by the *SD1* gene, we directly examined the intermediate GA levels in the *sd1* mutant (Table 1). The levels of GA_{20}, GA_1, GA_8 and GA_{29} in the *sd1* mutant were lower than in the wild-type, whereas the amount of GA_{53} in *sd1* was higher. GA_{44} and GA_{19} levels in *sd1* were equivalent to the levels detected in the wild-type. The activity of GA20ox, which catalyzes the three steps from GA_{53}-GA_{44}-GA_{19} to GA_{20} (Fig. 2b), was weaker in the mutant than in the wild-type, and therefore the *SD1* gene was considered to encode GA20ox.

Cloning and molecular characterization of sd1

One *GA20ox* gene (*GA20ox-1*) had already been isolated from rice (Toyomasu *et al.* 1997), but the *SD1* gene does not correspond to *GA20ox-1* because *GA20ox-1* has been mapped on chromosome 3 of the rice genome (data not shown) whereas *sd1* is located on the long arm of chromosome 1 (Maeda *et al.* 1997). Since the *Arabidopsis* genome carries three *GA 20-oxidase* genes and the products function in a redundant manner (Xu *et al.* 1995), we assumed that *SD1* might encode another GA20ox. In an attempt to isolate a novel *GA20ox* gene, we designed degenerate primers based on the conserved domain between the rice and *Arabidopsis GA20ox* genes (Toyomasu *et al.* 1997, Xu *et al.* 1995). Two amplified DNA fragments were obtained; one corresponded to the previously identified *GA20ox-1*, and the other was a novel *GA20ox* gene (*GA20ox-2*). Mapping analysis revealed that *GA20ox-2* was located on the long arm of chromosome 1, tightly linked to the *sd1* locus (Sasaki *et al.* 2002). The nucleotide (cDNA) sequence of *SD1* is shown in Figure 3. *GA20ox-2* gene has residues that bind active-Fe and interact with the 5-carboxylate of 2-oxoglutarate which are well conserved in GA20 oxidase. The deduced amino acid sequence of *GA20ox-2* showed a 47.8% identity to that of *GA20ox-1* and 49.5% identity to that of *Arabidopsis GA5* (Fig. 4). We cloned and sequenced the *GA20ox-2* genes from four *sd1* mutants and found that one *sd1* allele showd a 383 bp deletion (Dee-geo-woo-gen and IR8 have the same *sd1* allele) which induced a frame shift and created a stop codon, where as other three *sd1* alleles had single nucleotide substitutions which induced amino acid changes (Jikkoku, Reimei and Calrose76) (Fig. 3). A complementation test involving the introduction of the wild-type *GA20ox-2* gene into the *sd1* mutant resulted in the restoration of a normal height for the transgenic plants, confirming that the *sd1* mutant was due to a loss-of-function of the *GA20ox-2* gene (Fig. 5).

Table 1. Endogenous levels of various GAs in the wild and *sd1* plants

	GA_{53}	GA_{44}	GA_{19}	GA_{20}	GA_1	GA_8	GA_{29}
Wild	1.5[1)]	3.8	17	1.5	0.6	1.9	0.6
sd1	2.8	3.9	17	0.5	0.2	0.4	ND

[1)] GA levels of the wild type (Woo-gen) and *sd1* (Dee-geo-woo-gen) were measured by gas chromatography-mass spectrometry analysis.
nanograms/grams fresh weight

Biochemical analysis of the SD1 product

To demonstrate that *GA20ox-2* encodes an active GA20ox enzyme, we sub-cloned the coding region into an expression vector and expressed its product in *E. coli*. The recombinant protein catalyzed the conversion of GA_{53} to GA_{20} (Fig. 6). These results demonstrated that *GA20ox-2* encoded an active GA20 oxidase.

Expression analysis of the SD1 gene

We examined the expression pattern of *GA20ox-1* and *GA20ox-2* in leaves and flowers by RT-Southern blot analysis (Fig. 7a). *GA20ox-2* was expressed in both organs, leaves and flowers. In contrast, *GA20ox-1* was preferentially expressed in flowers. The expression of *GA20ox-2* in leaves corresponded well to the semi-dwarf phenotype of the *sd1* mutants. As reported previously (Sasaki *et al.* 2002), the presence of redundant enzymes in flowers could be important for fertilization of the *sd1* allele in rice breeding, since the loss-of-function of *SD1* may adversely affect flower development and fertilization if *SD1* were the sole gene encoding GA20ox in rice.

We also examined whether *GA20ox-2* is regulated by active GAs in a feedback manner, because the expression of some *GA20ox* genes is repressed by active GAs (Xu *et al.* 1999). For this study, we used germinating seeds in the presence of either GA_3 or the GA biosynthetic inhibitor, uniconazole. The expression of *GA20ox-2* was down-regulated by GA_3, indicating that the *GA20ox-2* gene is regulated by active GA (Fig. 7b).

Discussion

We have isolated and characterized the rice "green revolution" gene, *SD1*, and concluded that it encodes a GA biosynthetic enzyme, GA20ox, on the basis of the following results. Firstly, the *sd1* mutant responded to exogenous GA_3 and the level of endogenous GA_{20} in *sd1* was lower than that in the wild-type, whereas the level of GA_{53} in *sd1* was higher than that in wild-type plants. Secondly, each of the *sd1* mutants that we tested showed a different kind of mutation for the *GA20ox-2* gene. Thirdly, when the wild-type *GA20ox-2*

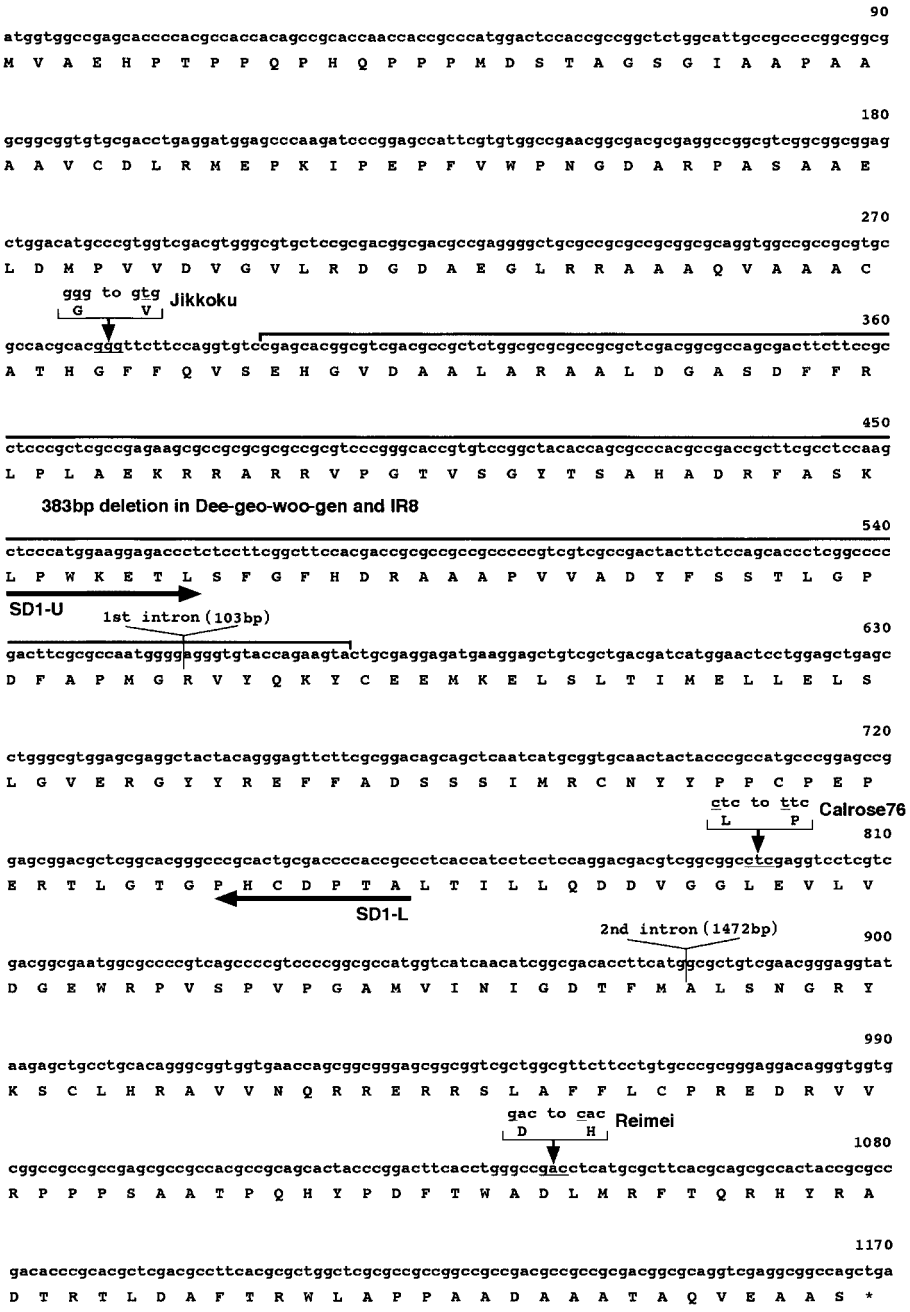

Fig. 3. Nucleotide (cDNA) and deduced amino acid sequences of *SD1*.
The position and the length of introns are indicated by vertical lines and numbers in parenthesis above the cDNA sequences, respectively. The position of mutations for the four *sd1* alleles is also indicated. The deleted sequence in Dee-geo-woo-gen is denoted by a hooked line and the single nucleotide substitutions in the three *sd1* alleles, Jikkoku, Calrose76 and Reimei, are indicated by vertical arrows. The corresponding sequences for the degenerate primers (SD1-U, SD1-L) are indicated by thick arrows. The accession number for *GA20ox-2* is: AB077025.

gene was introduced into the *sd1* plants, they showed the wild-type phenotype with normal height. Finally, the product of the *SD1* gene exhibited GA20 oxidase activity, catalyzing the conversion of GA_{53} to GA_{20} *in vitro*. In addition the expression of *GA20ox-2* was down-regulated by GA in a similar manner to that of some GA20oxs in other plants (Xu *et al.* 1999).

As mentioned above, several kinds of mutations in the *GA20ox-2* gene occurred in the *sd1* mutants, namely, a 383 bp deletion in Dee-geo-woo-gen and single nucleotide substitutions at different positions in Jikkoku, Calrose76 and Reimei. The mutation in the Dee-geo-woo-gen allele is

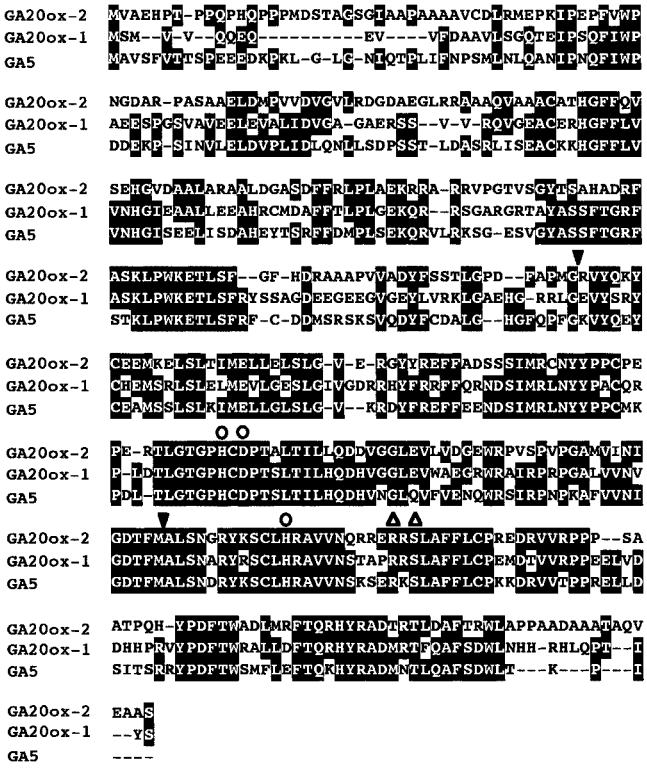

Fig. 4. Comparison of the deduced amino acid sequences of rice GA20ox-2 and GA20ox-1 and *Arabidopsis* GA5. Identical amino acids are indicated by the black boxes. Positions of introns are marked by arrowheads. Residues that bind active-Fe are indicated by ○. Residues that interact with the 5-carboxylate of 2-oxoglutarate are indicated by △.

likely to lead to a complete loss of GA20ox activity since it created a novel stop codon just after the deletion point. In contrast, the other three alleles showed a single nucleotide substitution which induced a single amino acid change. As the result, these mutations may only result in a partial rather than complete loss of the enzyme activity. In fact, the product of the Calrose76 allele retained some enzyme activity (data not shown).

The *sd1* alleles have been used in many breeding programs for both indica and japonica varieties. Koshio *et al.* (2000) compared the plant height of some isogenic lines of *sd1*. The isogenic line carried the *sd1* allele from Dee-geo-woo-gen lower than that had the *sd1* allele of Reimei (Koshio *et al.* 2000), suggesting that the Dee-geo-woo-gen *sd1* allele is stronger than that of Reimei. In general, since native indica varieties are taller than japonica varieties, the strong *sd1* allele has been used for indica breeding to develop semi-dwarf varieties such as IR8. On the other hand, weak *sd1* alleles have been selected for producing new japonica varieties, including Jikkoku, Calrose76 and Reimei. The results of our molecular analyses indicate that rice breeders recognized the variations of dwarfism among the various *sd1* alleles and used the most suitable allele for producing new varieties with the desired height.

A wheat semi-dwarf variety that was bred at CIMMYT

Fig. 5. Complementation test of the *sd1* mutant with the wild-type *GA20ox-2* allele. Right, A transgenic plant containing the wild-type *GA20ox-2* gene showed the normal phenotype. Left, A control transgenic plant containing the vector DNA.

(Wheat and Maize Improvement Center) also led to the wheat "green revolution" in the 1960s. Since both rice and wheat semi-dwarf varieties enabled to achieve major yield increases, semi-dwarfism is undoubtedly one of the most important agricultural traits for crop breeding. Recently, the wheat green revolution gene, *Rht* (reduced height), has been identified and it was found to encode a transcriptional factor that is a negative regulator of GA signaling (Peng *et al.*

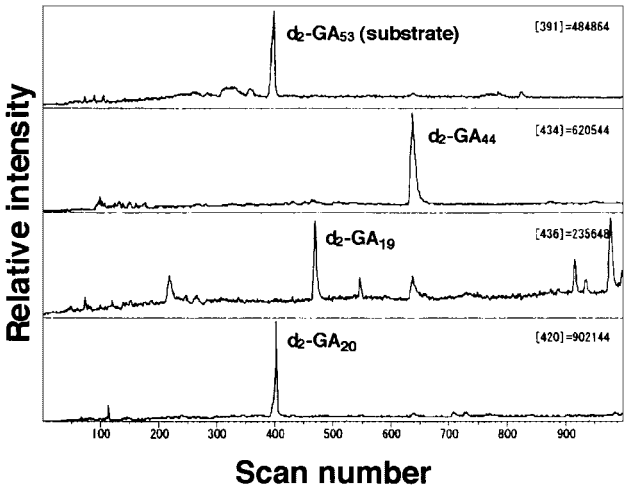

Fig. 6. Enzyme activity of GA20ox-2. Recombinant GA20ox-2 produced in *E. coli* catalyzed the steps from GA_{53} to GA_{20}. Deuterium-labeled GA_{53} (d_2-GA_{53}) was incubated with the recombinant GA20ox-2. In a full-scan GC-MS analysis, ions with m/e 391, 434, 436 and 420 were monitored for identification of d_2-GA_{53}, d_2-GA_{44}, d_2-GA_{19} and d_2-GA_{20}, respectively. The retention time of each peak was identical with that of authentic d_2-GA.

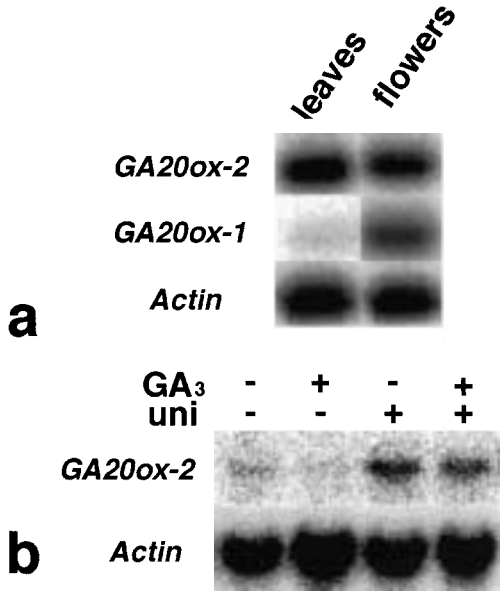

Fig. 7. Expression analysis of *GA20ox-2*
a. Expression of *GA20ox-2* in leaves and flowers. RT-PCR amplification was performed using the 1st strand cDNA and the products were detected by DNA blot analysis. Actin was used as a control.
b. Feedback repression of *GA20ox-2* by GA_3. uni, uniconazole.

1999). Because wheat has a hexaploid genome, a recessive allele such as *sd1* in rice could not be available for producing a semi-dwarf strain of wheat. Actually the *Rht* gene is a gain-of-function allele induced by a mutation in a specific region related to the perception of GA signaling (Peng *et al.* 1999). Even though the genetic and biochemical functions of the rice SD1 and wheat RHT proteins are completely different, that is, recessive versus dominant, loss-of-function versus gain-of-function events, enzyme versus transcriptional factor, respectively, the products of both genes are associated with GA. The fact that both "green revolution" genes are related to GA may not be a coincidence. Consequently, the manipulation of GA biosynthesis or perception may be a good target for regulating crop height.

The rate of world population growth has once again exceeded the rate of growth in food-grain production (Khush 1999), due to rapidly declining mortality rates from advancements in modern medicine and health care and a reduction in arable land. And now the impending food crisis is a cause for concerned. To meet the global food demand, grain production will have to increase by 50% by 2025 (Khush, 1999). Prompt measures and action for a second green revolution have been called for to avoid widespread food shortages in the future. The marker assisted selection using *sd1* gene as selection marker is useful for efficient breeding program. As our present study revealed that modulating active GA levels during the vegetative stage can lead to a suitable plant architecture for high crop yield, the genetic manipulation of GA-biosynthesis using a molecular biology approach may provide us with an opportunity to address food security concerns, as was the case for the green revolution in the 1960s.

Acknowledgements

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Added in proof

After we submitted the manuscript, a paper entitled "Positional Cloning of Rice Semi-dwarfing Gene, *sd-1*: Rice Green Revolution Gene Encodes a Mutant Enzyme Involved in Gibberellin Synthesis" was published (DNA Research. Vol. 9, Page 11-17). The paper suggested that the *SD1* gene encodes a GA20ox enzyme by comparison of the nucleotide sequences between wild and mutant alleles.

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