Cloning and functional analysis of two gibberellin 3β-hydroxylase genes that are differently expressed during the growth of rice

Hironori Itoh*, Miyako Ueguchi-Tanaka*, Naoki Sentoku*, Hidemi Kitano†, Makoto Matsuoka*, and Masatomo Kobayashi‡§

*Bioscience Center and †Graduate School of Bioagricultural Sciences, Nagoya University, Chikusa, Nagoya, 464-8601, Japan; and ‡RIKEN Tsukuba Institute, Tsukuba, Ibaraki 305-0074, Japan

Communicated by Bernard Phinney, University of California, Los Angeles, CA, May 14, 2001 (received for review October 28, 1999)

We have cloned two gibberellin (GA) 3β-hydroxylase genes, *OsGA3ox1* and *OsGA3ox2*, from rice by screening a genomic library with a DNA fragment obtained by PCR using degenerate primers. We have used full-scan GC-MS and Kovats retention indices to show function for the two encoded recombinant fusion proteins. Both proteins show 3β-hydroxylase activity for the steps GA_{20} to GA_1, GA_5 to GA_3, GA_{44} to GA_{38}, and GA_9 to GA_4. In addition, indirect evidence suggests that the OsGA3ox1 protein also has 2,3-desaturase activity, which catalyzes the steps GA_9 to 2,3-dehydro-GA_9 and GA_{20} to GA_5 (2,3-dehydro GA_{20}), and 2β-hydroxylase activity, which catalyzes the steps GA_1 to GA_8 and GA_4 to GA_{34}. Molecular and linkage analysis maps the *OsGA3ox1* gene to the distal end of the short arm of chromosome 5; the *OsGA3ox2* gene maps to the distal end of the short arm of chromosome 1 that corresponds to the *D18* locus. The association of the *OsGA3ox2* gene with the *d18* locus is confirmed by sequence and complementation analysis of three *d18* alleles. Complementation of the *d18-AD* allele with the *OxGA3ox2* gene results in transgenic plants with a normal phenotype. Although both genes show transient expression, the highest level for *OsGA3ox1* is from unopened flower. The highest level for *OsGA3ox2* is from elongating leaves.

Gibberellins (GAs) are a large family of tetracyclic, diterpenoid plant hormones that promote various growth and developmental processes in higher plants. A number of GA-responsive dwarf mutants have been isolated from various plant species, including maize (*Zea mays*), pea (*Pisum sativum*), tomato (*Lycopersicon esculentum*), thale cress (*Arabidopsis thaliana*), and rice (*Oryza sativa*) (1–7). For example, in maize the *d1* mutant is rescued by the exogenous application of GA_1, but not by GA_{20}, the immediate precursor of GA_1. The mutant accumulates GA_{20} and metabolizes GA_{20} to GA_1 at a low rate (8, 9). Presumably the *D1* gene encodes a 3β-hydroxylase that controls this step. In pea the *LE* gene has been cloned and shown to encode a GA 3β-hydroxylase (10, 11). The *le* mutant is rescued by GA_1, but not by GA_{20}, and shows low activity in the metabolism of GA_{20} to GA_1 (12, 13).

For rice the *d18* mutant has been characterized as a GA-responsive dwarf (5), and three alleles have been identified (2, 5). They are the two strong alleles, *d18-Id18*^{h} (Hosetsu-waisei dwarf) and *d18-AD* (Akibare-waisei dwarf), and the weak allele, *d18-dy* (Waito-C). The strong alleles promote severe dwarf phenotypes; the weak allele promotes a semidwarf phenotype. Physiological and biochemical studies have been carried out with the *d18-dy* mutant only. The dwarf phenotype of the *d18-dy* mutant is rescued by the application of GA_1, not by GA_{20} (2). Seedlings of this mutant are deficient in GA_1 and accumulate its immediate precursor, GA_{20} (14). The metabolism of GA_{20} to GA_1 is lower in *d18-dy* plants than in normal ones (15). It has been proposed that the *D18* gene encodes a GA 3β-hydroxylase (15).

Studies have shown that GAs also regulate the development of reproductive organs. For example, a GA-deficient mutant in *Arabidopsis*, *ga1–3*, exhibits a male-sterile phenotype (4), and in tomato *stamenless-2* and *gib-1* arrest anther development at an early stage (16, 17). In tall rice, it has been reported that GA_4 accumulates in anthers (18) and that a cell-free extract from anthers metabolizes GA_{12} to GA_{34} and GA_{53} to GA_8 (19). The level of GA_4 and the activity of GA 3β-hydroxylase from anthers were the same for *d18-dy* mutants and normals, suggesting that there are at least two GA 3β-hydroxylase genes in rice (14, 19).

Although GA 3β-hydroxylase genes have been recently cloned from several dicot species (10, 11, 20–24), there is no example from monocots. In this study, we report the cloning from rice of two genes, *OsGA3ox1* and *OsGA3ox2*. *OsGA3ox1* is a new gene found on chromosome 5. *OsGA3ox2* is shown to correspond to the *D18* locus, which is known to be located on chromosome 1.

PLANT BIOLOGY

Materials and Methods

Plant Materials. Seeds of Japonica-type rice *(Oryza sativa* L.), tall cultivars Nipponbare and Akibare, and dwarf cultivars Akibare-waisei (*d18-AD*), Hosetsu-waisei (*d18-ID18*^{h}), and Waito-C (*d18-dy*) were surface-sterilized in 1% NaClO for 1 h and then rinsed in sterile distilled water. The seeds were planted in soil and grown in a greenhouse or paddy field. For RNA gel blot analysis, the tissues and organs were collected from tall rice (Akibare). Samples were collected from the following material: young shoots 2 weeks after planting, shoot apices, leaf blades, and elongating leaves (2–3 cm length, no boundary between sheath and blade) 1 month after planting, young panicles (1–2 cm length) approximately 1 week after panicle initiation, stems (nodes plus internodes) at internode elongation stage, and rachises and unopened flowers just before heading stage. Flowers of tall rice (Nipponbare) also were collected at the following anther developmental stages: differentiation stage of pollen mother cells, early stage of pollen differentiation, middle stage of pollen differentiation, late stage of pollen differentiation, and pollen maturing stage. Palea and lemma were removed from the flowers to give anthers and ovaries that were used for RNA gel blot analysis. Flowers at the late stage of pollen differentiation also were used for *in situ* hybridization. The seeds of rice used for mapping of recombinant inbred lines between the cultivars Asominori (Japonica type) and IR-24 (Indica type) were kindly provided by A. Yoshimura, Kyushu University (Fukuoka, Japan).

Abbreviation: GA, gibberellin.

Data deposition: The nucleotide sequences reported in this paper have been deposited in the GenBank database [accession nos. AB054084 for the *OsGA3ox1* gene, AB056519 for the *OsGA3ox2*(*D18*) gene, AB056517 for the *d18-Id18*^{h} gene, and AB056518 for the *d18-dy* gene].

§To whom reprint requests should be addressed at: RIKEN Tsukuba Institute, 3-1-1 Koyadai, Tsukuba, Ibaraki 305-0074, Japan. E-mail: kobayasi@rtc.riken.go.jp.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

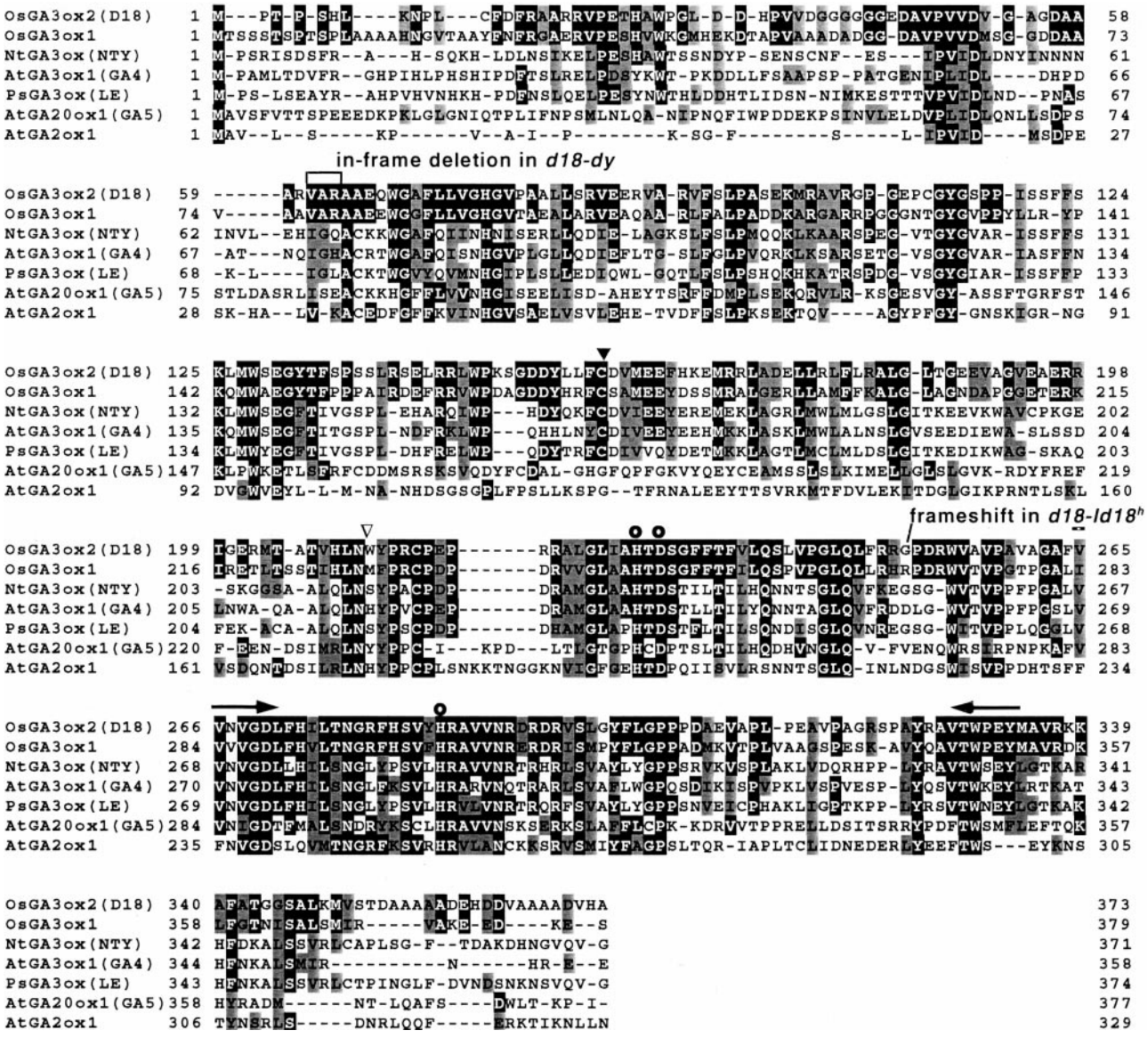

Fig. 1. Alignment of deduced amino acid sequences of GA 3β-hydroxylases and other GA dioxygenases from the monocot, rice, and several dicots. Alterations in the sequences from *d18-Id18*^{h} and *d18-dy* are indicated. Light/dark shading indicates similar/identical residues. The three residues that are invariant throughout the plant 2-oxoglutarate-dependent dioxygenases (whose side chains act as iron ligands) are marked with ○. ▼ indicates the position of the common intron in the rice 3β-hydroxylase genes; ▽ indicates the second intron in the *OsGA3ox1* gene. Dashes indicate gaps introduced to optimize alignment. Arrows indicate the regions used in the design of degenerate primers. GenBank accession numbers are as follows: OsGA3ox1, AB054084; OsGA3ox2, AB056519; NtGA3ox, AB032198; AtGA3ox1, L37126; PsGA3ox, AF001219; AtGA20ox1, X83379; AtGA2ox1, AJ132435. Nucleotide sequences of *OsGA3ox2* for *d18-Id18*^{h} (accession number AB056517) and *d18-dy* (accession number AB056518) mutants are in GenBank. Recently, the nucleotide sequence of a P1 artificial chromosome clone that harbors *OsGA3ox2* gene is registered in GenBank (accession number AP002523).

Chemicals. [17,17-$^2H_2$]GA_{19}, [17,17-$^2H_2$]GA_{20}, [17,17-$^2H_2$]GA_{29}, [17,17-$^2H_2$]GA_{44}, and [17,17-$^2H_2$]GA_{53} were purchased from L. Mander, Australian National University (Canberra). [15,17,17-$^2H_3$]GA_9 was synthesized from GA_9-norketone and (methyl-d_3)triphenylphosphonium-Br by Wittig reaction. All GAs used in this study were analyzed by full-scan GC-MS to show the absence of impurities. Uniconazole, an inhibitor for GA biosynthesis, was obtained from Sumitomo Chemical Company (Tokyo).

GC-MS. GC-MS was performed with an Auto Mass mass spectrometer (JEOL) connected to a Hewlett–Packard 5890 series II gas chromatograph. The analytical conditions used are described by Kobayashi *et al.* (25).

Genomic DNA Preparation. The genomic DNA used for PCR and Southern hybridization was prepared from the seedlings of tall rice (Nipponbare) using the methods described by Murray and Thompson (26). Genomic DNA also was prepared from *d18-Id18h*, *d18-dy*, and *d18-AD* plants by the same method used for the characterization of the *d18* alleles.

Isolation of cDNA Clones Encoding Putative GA 3β-Hydroxylases. The PCR was performed by using genomic DNA from Nipponbare as a template and degenerate primers (sense, 5′-GTNGT-NAAYGTNGGNGAYRT-3′, and antisense, 5′-TRRTAYT-CRTTCCANGTNAC-3′, indicated by arrows in Fig. 1). The reaction mixture (30 μl) contained 100 ng genomic DNA (Nipponbare), 1× PCR buffer (Applied Biosystems), 1.5 mM $MgCl_2$, 200 μM dNTP, 2 μM each primer, 1.5 μl DMSO, and 1 unit of *Taq* polymerase (Amplitaq, Applied Biosystems). The reactions were heated to 99°C for 10 min, and then 35 cycles of

amplification were performed (96°C for 1 min, 50°C for 2 min, and 72°C for 1 min) followed by a final 5-min incubation at 72°C. The 210-bp DNA fragment obtained was sequenced and used as a probe for screening a rice genomic library (constructed from Nipponbare and IR-36), resulting in the isolation of clones *OsGA3ox1* and *OsGA3ox2*, that contain the entire lengths of putative GA 3β-hydroxylase genes. Full-length cDNA clones for *OsGA3ox1* and *OsGA3ox2* were obtained by reverse transcription–PCR from the total RNA prepared from rice (Nipponbare) shoot apices and unopened flowers using methods described by Sambrook *et al.* (27). Reaction mixture (30 μl) for reverse transcription contained 1 μg total RNA, 5 mM $MgCl_2$, 1× PCR buffer, 1 mM dNTP, 0.125 μM oligo(dT) primer, and AMV Reverse Transcriptase XL (RNA-PCR kit, AMV version 2.1, Takara, Kyoto, Japan). The reverse transcription was carried out at 45°C for 1 h, followed by PCR in which 2 μl of the reaction mixture from reverse transcription was used as a template. Other conditions in the PCR were as described above for degenerate primers. The following oligonucleotide primers were used in the PCR: for *OsGA3ox1*, sense, 5′-CCGGATCCATGACATCGTCGTCGACCTCGCCG-3′ and antisense, 5′-GGAAGCTTCTAACTCTCCTTGTCCTCTTC-3′; for *OsGA3ox2*, sense, 5′-CCGGATCCATGCCGACGCCGTCGCACTTGAA-3′ and antisense 5′-GGAAGCTTCTATAGCTTATGCGTGGACGT-3′.

Sequence Analysis. Nucleotide sequences were determined by the dideoxynucleotide chain-termination method using an automated sequencing system (model ABI373A, Applied Biosystems). Sequences were analyzed with GENETYX computer software (Software Development, Tokyo).

Heterologous Expression and Enzyme Assays. The entire coding region of the *OsGA3ox1* and *OsGA3ox2* cDNA were inserted into a suitable restriction site of pMAL-c2 and transformed into *Escherichia coli* strain JM109. A fresh overnight culture (1 ml) of *E. coli* was added to 100 ml of 2× YT medium [NaCl (1 g/liter), yeast extract (1 g/liter), and tryptone (1.6 g/liter)] containing ampicillin (0.1 mg/liter). The culture was incubated at 30°C for 4 h. Isopropyl β-D-thiogalactoside (1 mg/liter) was added, and the culture was further incubated at 17°C for 18 h. The cells were harvested, homogenized by sonication, and centrifuged to give a crude extract. The presence of the OsGA3ox1 recombinant protein in the crude extract was confirmed by SDS/PAGE, and this crude extract was used in the enzyme assay. Because much less amount of the *OsGA3ox2* recombinant protein was observed in the crude extract, it was concentrated by an affinity column packed with 100 μl amylose resin according to the manufacturer's instruction (New England Biolabs). The eluate from the column was used in the enzyme assay; 2-ketoglutarate (final concentration, 5 mM), ascorbate (5 mM), and $FeSO_4$ (0.5 mM) were added to the eluate (200 μl) to give a reaction mixture, and it was incubated with substrate GA at 30°C for 1 h. The reaction mixture was adjusted to pH 2 with HCl and extracted with ethyl acetate. The extract was analyzed by full-scan GC-MS and Kovats retention indices to identify product GAs (25).

DNA and RNA Gel Blot Analyses. Gene-specific probes were prepared by α-[^{32}P]dCTP labeling of a *Kpn*I–*Pvu*II fragment (369 bp) of *OsGA3ox1* cDNA and a *Bss*HII–*Pvu*II fragment (429 bp) of *OsGA3ox2* cDNA according to the manufacturer's instruction (Ready-to-Go DNA Labeling Beads, Amersham Pharmacia). Each specific probe was used in genomic Southern blot hybridization to confirm the absence of cross-hybridization. Rice genomic DNA (from Nipponbare; 1 μg per lane) was digested with 10 units of *Eco*RV (for *OsGA3ox1* probe) or *Apa*I (for *OsGA3ox2* probe) at 37°C overnight, separated by 0.7% agarose gel electrophoresis, and transferred to Hybond N^+ nylon membrane (Amersham Pharmacia) (27). Hybridization was performed at 65°C in 0.25 M Na_2HPO_4, 1 mM EDTA, and 7% SDS. Filters were washed twice in 2× SSC, 0.1% SDS at 65°C for 15 min, and once in 0.2× SSC, 0.1% SDS at 65°C for 15 min.

In RNA gel blot analyses, total RNA was prepared as described by Sambrook *et al.* (27). RNA (10 μg per sample) was electrophoresed and transferred to Hybond N^+ nylon membrane. Hybridization was performed in 5× SSC, 10% (wt/vol) dextran sulfate, 0.5% (wt/vol) SDS, and 0.1 mg/ml denatured salmon sperm DNA at 65°C. Filters were washed with the procedure mentioned above.

To investigate the effects of uniconazole and GA_3 on the expression of the rice GA 3β-hydroxylase genes, seeds of Nipponbare were sterilized, as described above, placed in Petri dishes, and imbibed in 10 ml of water. An ethanol solution (100 μl) of either uniconazole (final concentration, 10 μM) or GA_3 (final concentration, 10 μM) was added to the culture. For a control experiment, only ethanol (100 μl) was added to the culture. Twenty-four hours later, the total RNA was isolated from the embryos.

Characterization of the *d18* Alleles. Genomic DNA was prepared from the *d18* dwarf mutants (*d18-Id18^h*, *d18-dy*, and *d18-AD*) and used for PCR as a template to amplify the 1.6-kb fragment that include the entire coding region of *OsGA3ox2*. The fragments obtained from the *d18-Id18^h* (Hosetsu-waisei) and *d18-dy* (Waito-C) alleles were sequenced.

Complementation Analysis. A binary vector, pBI-Hm, was prepared from pBI-H1 (kindly provided by K. Nakamura, Nagoya University, Nagoya, Japan) by incorporation of the hygromycin-resistant gene into pBI-H1. A genomic fragment (8 kbp) harboring the *OsGA3ox2* locus was cloned into the *Hin*dIII site of the pBI-Hm. The binary vector was introduced into *Agrobacterium tumefaciens* EHA101 (28) by electroporation. Transformation of rice with *A. tumefaciens* was performed as described by Hiei *et al.* (29).

***In Situ* Hybridization.** Flowers were fixed overnight at 4°C in 4% (wt/vol) paraformaldehyde plus 0.25% (vol/vol) glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.2). The fixed material was dehydrated by a graded concentration of ethanol, then *t*-butanol, and embedded in Paraplast Plus (Sherwood Medical, Norfolk, NE). Microtome sections (9 μm thick) were mounted on silanized glass slides (Matsunami Glass, Osaka, Japan). Paraffin was removed by using a graded concentration of ethanol, and samples were dried for 1 h.

In situ hybridization was performed by using digoxygenin-labeled sense or antisense RNA prepared from cDNA of *OsGA3ox1* and *OsGA3ox2*. Hybridization and detection of the probes were performed by using methods described by Kouchi and Hata (30).

Results and Discussions

Isolation of GA 3β-Hydroxylase Genes. Using rice genomic DNA as a template, we performed PCR with degenerate primers to give a DNA fragment that had relatively high homology with previously reported GA 3β-hydroxylase genes (10, 11, 20–24). A rice genome library was screened with this fragment as a probe to isolate full-length clones. Sequence analysis of the clones revealed the occurrence of two genes for putative GA 3β-hydroxylase. They were designated as *OsGA3ox1* and *OsGA3ox2* according to the nomenclature used by Coles *et al.* (31).

Based on the sequences of the clones, reverse transcription–PCR was performed to obtain the cDNA clones. Full-length cDNA fragments of *OsGA3ox1* and *OsGA3ox2* contained an ORF encoding polypeptides of 379 and 373 aa, respectively (Fig. 1). The deduced amino acid sequences of both clones suggested that they belonged to a family of 2-oxoglutarate-dependent

Table 1. Identification of the metabolites from GAs incubated with recombinant rice GA 3β-hydroxylases by full-scan GC-MS and Kovats retention indices (KRI)

Recombinant protein	Substrate GA	Product GA*	KRI	Characteristic ions at m/z (% relative intensity of base peak)
OsGA3ox1	$^2H_3$-GA_9	GA_4	2610	479 (17), 464 (31), 389 (36), 227 (100)
		GA_7	2627	477 (4), 462 (11), 389 (6), 225 (100)
		GA_{34}	2750	567 (100), 552 (14), 477 (4), 232 (18)
	$^2H_2$-GA_{20}	GA_1	2748	566 (100), 551 (31), 449 (33), 209 (25)
		GA_3	2766	564 (100), 549 (42), 447 (26), 210 (48)
		GA_8	2880	654 (13), 639 (5), 537 (100), 313 (11)
	$^2H_0$-GA_5	GA_3	2768	562 (100), 547 (47), 445 (38), 208 (79)
	$^2H_2$-GA_{44}	GA_{38}	2939	522 (73), 507 (6), 432 (5), 209 (100)
OsGA3ox2	$^2H_3$-GA_9	GA_4	2608	479 (12), 464 (21), 389 (25), 227 (100)
	$^2H_2$-GA_{20}	GA_1	2745	566 (100), 551 (30), 449 (60), 209 (45)
	$^2H_0$-GA_5	GA_3	2766	562 (87), 547 (38), 445 (48), 208 (100)
	$^2H_2$-GA_{44}	GA_{38}	2940	522 (84), 507 (8), 432 (4), 209 (100)

*For the identification of GA_{38}, the sample was derivatized to the methyl ester trimethylsilyl ether. Other samples were trimethylsilylated.

dioxygenases. Such dioxygenases contain sequences that are conserved within the class of enzyme, including two histidine and one asparatic acid residues at cofactor binding sites. Their sequences had the greatest similarity to GA 3β-hydroxylase sequences of enzymes that catalyze the later steps of GA biosynthesis (Fig. 1). However, the degree of similarity was less than those observed among dicot GA 3β-hydroxylases. Like other GA 3β-hydroxylases, *OsGA3ox2* had one intron in its coding region, whereas *OsGA3ox1* had two introns.

Functional Analyses. To demonstrate that *OsGA3ox1* and *OsGA3ox2* encode active GA 3β-hydroxylases, we subcloned the coding regions in expression vector pMAL-c2 to produce in-frame fusion proteins and expressed them in *E. coli*. The recombinant proteins were used for functional assays, and the results are summarized in Table 1. Both recombinant proteins catalyzed the conversions of GA_5 to GA_3, GA_9 to GA_4, GA_{20} to GA_1 and GA_{44} to GA_{38}. The metabolic step of GA_5 to GA_3 indicates a 3β-hydroxylation with rearrangement of double bond. The metabolism of GA_5 to GA_3 has been observed in rice (32). In maize, the *dwarf-1* gene (putative GA 3β-hydroxylase) was suggested to catalyze this step (33). Thus, it is not surprising that the rice GA 3β-hydroxylase can catalyzes the metabolism of GA_5 to GA_3. In addition, it was suggested that OsGA3ox1 protein catalyzes 2,3-desaturation and 2β-hydroxylation. For example, GA_9 was metabolized not only to GA_4, but also to GA_7 and GA_{34} (Fig. 2). As determined by total ion current, GA_4 occupied 65% of the total amount of metabolites, whereas 29% was occupied for GA_7 and 6% for GA_{34}. The identification of GA_7 suggests that the OsGA3ox1 protein has 2,3-desaturation activity (34); this 2,3-desaturation activity is consistent with the fact that GA_7 has been identified in rice anthers (35). The identification of GA_{34} revealed that OsGA3ox1 protein also catalyzes 2β-hydroxylation. No metabolite was identified from GA_{53}, GA_{19}, and GA_{29}. These results show that *OsGA3ox2* encodes an active GA 3β-hydroxylase and that *OsGA3ox1* encodes a novel type of GA 2β,3β-hydroxylase, whose substrate specificity is different from pumpkin GA 2β,3β-hydroxylase (21). It is noteworthy that partially purified GA 3β-hydroxylase from bean catalyzed 2β-hydroxylation of GA_{20} to produce GA_{29} (36). However, the 2β-hydroxylated GAs, namely GA_{34} from GA_9 and GA_8 from GA_1, were minor products of the reactions, and the role of 2β-hydroxylation activity of OsGA3ox1 protein in growth regulation is unclear.

Genetic Analysis. The *D18* locus maps to the top of the short arm of chromosome 1, flanking the *FS-2* locus (37, 38). To test whether *OsGA3ox1* and/or *OsGA3ox2* maps to the *D18* locus, we

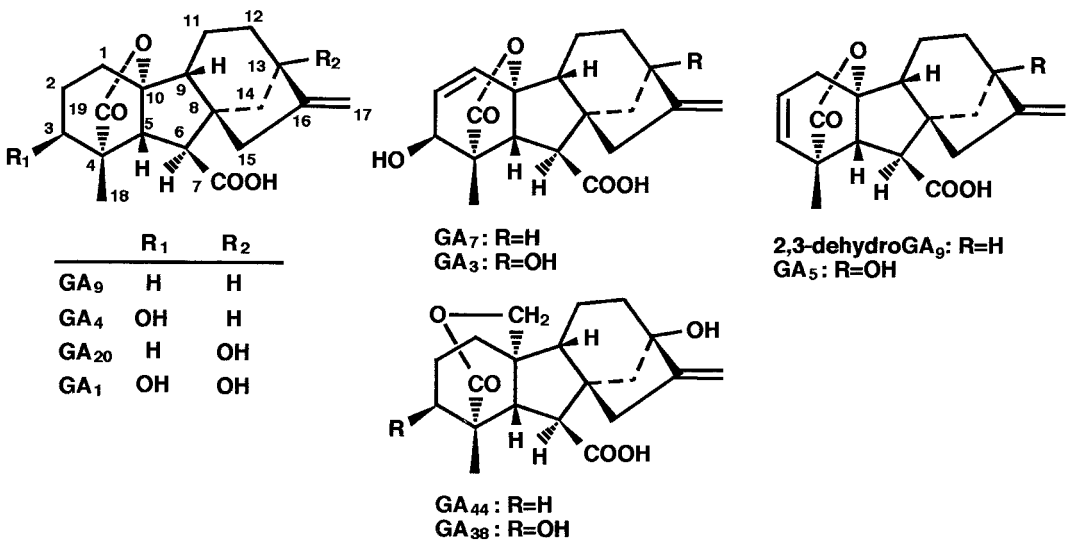

Fig. 2. Numbering system and structures of the four GAs used in the metabolic studies. See Table 1 for specific feeds and metabolites.

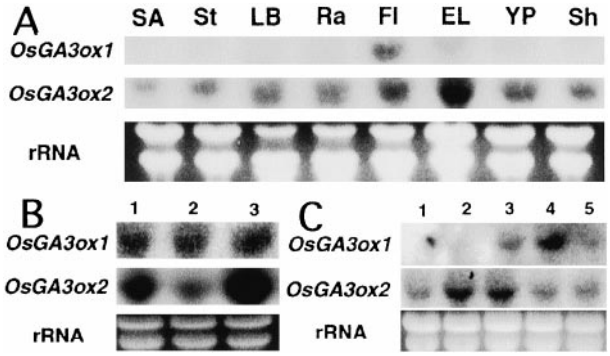

Fig. 3. (*A*) RNA gel blot analyses of *OsGA3ox1* and *OsGA3ox2* in various rice organs (cultivar Akibare). Total RNA was isolated from shoot apices (SA), stems (St), leaf blades (LB), rachis (Ra), unopened flowers (Fl), elongating leaves (EL), young panicles (YP), and shoots (Sh). (*B*) Effects of GA_3 and uniconazol on the expression of *OsGA3ox1* and *OsGA3ox2* in germinating seeds. Lane 1, control; lane 2, 10 μM GA_3; lane 3, 10 μM uniconazole. (*C*) Gene expression of GA 3β-hydroxylases in anthers and ovaries at different stages of development. Lane 1, stage differentiation of pollen mother cells; lane 2, early-stage pollen differentiation; lane 3, middle-stage pollen differentiation; lane 4, late-stage pollen differentiation; lane 5, mature-stage pollen. Blots were hybridized with probes for GA 3β-hydroxylases (*OsGA3ox1, OsGA3ox2*). Approximately 10 μg of total RNA from each sample was loaded onto the gel and stained with ethidium bromide (rRNA).

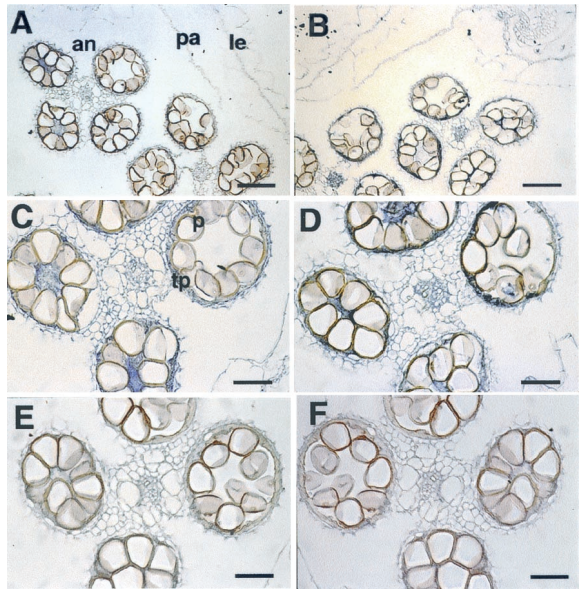

Fig. 4. *In situ* localization of *OsGA3ox1* (*A*, *C*, and *E*) and *OsGA3ox2* (*B*, *D*, and *F*) mRNA during the stage of late pollen differentiation (same stage as in Fig. 3*C*, lane 4). (*A* and *B*) Cross section of a flower hybridized with the antisense probe. The signal (blue staining) can be seen in the anthers. (*C* and *D*) Higher-magnification view of a cross section of anthers stained with the antisense probes. Note the staining in the tapetum. (*E* and *F*) Anthers at the same stage, stained with the sense probe. Morphological features of tissues: an, anther; pa, palea; le, lemma; p, pollen; tp, tapetum. (Bar indicates 50 μm in *A* and *B*; 100 μm in *C–F*.)

performed restriction fragment length polymorphism analysis. DNA gel blot analysis with gene-specific probes for *OsGA3ox1* and *OsGA3ox2* revealed *Eco*RV (*OsGA3ox1*) and *Apa*I (*OsGA3ox2*) polymorphisms between Asominori and IR 24. Linkage analysis was performed by using recombinant inbred lines prepared by crossing of Asominori and IR 24 (39). *OsGA3ox1* mapped to the distal end of the short arm of chromosome 5; *OsGA3ox2* mapped to the distal end of the short arm of chromosome 1. The mapping position of *OsGA3ox1* was between R3166 (18.5 centimorgans, cM) and C119 (27.5 cM) on chromosome 5, whereas that of *OsGA3ox2* was between R1613 (16.1 cM) and R1944 (24.1 cM) on chromosome 1. Thus *OsGA3ox2* corresponds to the *D18* locus.

To confirm the relation of *OsGA3ox2* to the *D18* locus, we directly analyzed the entire coding sequences of *OsGA3ox2* isolated from *d18* alleles and compared the sequences with the wild type (Nipponbare) (Fig. 1). In the strong allele, *d18-Id18*^{h}, deletion of a guanine base at position 750 (counted from the adenine in the start codon) shifts the reading frame. In the weak allele, *d18-dy*, an in-frame 9-base deletion at positions 181–189 deleted three aa resides, Val^{61} to Arg^{63}. In a strong allele, *d18-AD*, no PCR product was generated. Genomic Southern analysis of *d18-AD* indicated the occurrence of 7-kbp deletion in the genomic DNA fragment that include *D18* locus. Based on this evidence, it was concluded that at least a part of the coding sequence of *OsGA3ox2* was lost in the *d18-AD* mutant. Further analysis has not been done on this allele. We also carried out the complementation of *d18-AD* by the introduction of the wild-type *OsGA3ox2* gene (8.2 kbp) containing 5′ and 3′ flanking sequences (6 kbp and 1 kbp), all exons (1.1 kbp), and an intron (0.1 kbp). A normal phenotype appeared in eight of 10 transgenic plants. These results confirmed that *OsGA3ox2* corresponds to the *D18* locus. The *in vitro* assay with GA_{20} as a substrate showed that d18-ld18^h protein had no activity; a slight activity was observed from the d18-dy protein (data not shown).

Gene Expression. The expression of *OsGA3ox1* and *OsGA3ox2* was examined in several organs using RNA gel blot analysis with gene-specific probes (Fig. 3*A*). The expression of *OsGA3ox1* was highest in unopened flowers. The expression of *OsGA3ox2* was highest in elongating leaves and absent in shoot apices.

It has been reported that the expression of *GA4* (*AtGA3ox1*) in *Arabidopsis* and *LE* (*PsGA3ox1*) in pea is under feedback regulation (11, 20). We used germinating seeds to examine the expression of *OsGA3ox1* and *OsGA3ox2* in the presence of GA_3 and the GA biosynthetic inhibitor, uniconazol (Fig. 3*B*). Both *OsGA3ox1* and *OsGA3ox2* were expressed in the germinating seed (Fig. 3*B*, lane 1). Neither GA_3 nor uniconazole affected the expression level of *OsGA3ox1*. The expression of *OsGA3ox2* was down-regulated by GA_3 (Fig. 3*B*, lane 2) and up-regulated by uniconazole (Fig. 3*B*, lane 3).

Kobayashi *et al.* (19) have reported that mature anthers of rice metabolize GAs. We have examined the expression of *OsGA3ox1* and *OsGA3ox2* in the anthers and ovaries at five developmental stages (Fig. 3*C*). The level of the *OsGA3ox1* transcript was high at late-stage pollen differentiation only, whereas the level of the *OsGA3ox2* transcript was relatively high at early-stage pollen differentiation and middle-stage pollen differentiation.

To investigate the expression site of *OsGA3ox1* and *OsGA3ox2* in the anther, *in situ* hybridization was performed with digoxygenin-labeled RNA probes. Blue staining by an antisense probe for *OsGA3ox1* and *OsGA3ox2* were observed in the tapetum of anthers (Fig. 4 *A–D*). No staining was observed with the sense-strand probes (Fig. 4 *E* and *F*), which indicates that the blue staining by antisense probes was not an artifact.

Although *OsGA3ox1* and *OsGA3ox2* share high homology in their deduced amino acid sequences, their expression patterns and *in vitro* activities are different from each other. We show that *OsGA3ox2* corresponds to the *D18* gene, a gene that is known to control the elongation of the vegetative shoot. The feedback regulation of the expression of *OsGA3ox2* supports this position. The relatively high level of expression of *OsGA3ox2* in immature flower tissues suggests that *OsGA3ox2* also may be involved in

the regulation of the growth of reproductive tissues. Several reports suggest that GA biosynthesis is required for anther development (4, 16, 17, 40). However, the *d18* mutants are not male-sterile, which suggests that *OsGA3ox2* may have a limited role in the regulation of anther development.

OsGA3ox1 was expressed mainly in the tapetum at late-stage pollen differentiation. Although *OsGA3ox2* also was expressed in the tapetum at this stage, the expression level was relatively low. These observations suggest that the *OsGA3ox1* gene is responsible for GA_4 biosynthesis in the anthers, which would explain the previous observation that GA_4 accumulates in the anthers of *d18-dy* plant (14). Hasegawa *et al.* (41) studied the localization of GA_4 in anthers by immunohistochemistry and found that GA_4 apparently accumulated in the tapetum and pollen grains. The expression of *OsGA3ox1* and *OsGA3ox2* in the tapetum partially explains their results, although the metabolic origin of the GA_4 observed in pollen grains is unknown.

We thank Dr. Soon-Kwan Hong for his advice and help on *in situ* hybridization experiments. This work was supported by the program Isolation and Functional Analysis of Useful Rice Genes and Development of Techniques for their Utilization from the Ministry of Agriculture, Forestry and Fishery, and by the Special Coordination Funds for Promoting Science and Technology from Research Fellowship of the Japan Society for the Promotion of Young Scientists (to H.I.).

1. Phinney, B. O. (1956) *Proc. Natl. Acad. Sci. USA* **42,** 185–189.
2. Murakami, Y. (1972) in *Plant Growth Substances 1970***,** ed. Carr, D. J. (Springer, Berlin), pp. 166–174.
3. Koornneef, M. (1978) *Arabidopsis Inf. Serv.* **15,** 17–20.
4. Koornneef, M. & van der Veen, J. H. (1980) *Theor. Appl. Genet.* **58,** 257–263.
5. Kinoshita, T. & Shinbashi, N. (1982) *Jpn J. Breed.* **32,** 219–231.
6. Koornneef, M., Bosma, T. D. G., Hanhart, C. J., van der Veen, J. H. & Zeevaart, J. A. D. (1990) *Theor. Appl. Genet.* **80,** 852–857.
7. Reid, J. B. & Ross, J. J. (1993) *Int. J. Plant Sci.* **154,** 22–34.
8. Spray, C., Phinney, B. O., Gaskin, P., Gilmour, S. J. & MacMillan, J. (1984) *Planta* **160,** 464–468.
9. Fujioka, S., Yamane, H., Spray, C. R., Gaskin, P., MacMillan, J., Phinney, B. O. & Takahashi, N. (1988) *Plant Physiol.* **88,** 1367–1372.
10. Martin, D. N., Proebsting, W. M. & Hedden, P. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 8907–8911.
11. Lester, D. R., Ross, J. J., Davies, P. J. & Reid, J. B. (1997) *Plant Cell* **9,** 1435–1443.
12. Ingram, T. J., Reid, J. B., Potts, W. C. & Murfet, I. C. (1983) *Physiol. Plant* **59,** 607–616.
13. Ingram, T. J., Reid, J. B., Murfet, I. C., Gaskin, P., Willis, C. L. & MacMillan, J. (1984) *Planta* **160,** 455–463.
14. Kobayashi, M., Sakurai, A., Saka, H. & Takahashi, N. (1989) *Plant Cell Physiol.* **30,** 963–969.
15. Kobayashi, M., Gaskin, P., Spray, C. R., Phinney, B. O. & MacMillan, J. (1994) *Plant Physiol.* **106,** 1367–1372.
16. Sawhney, V. K. & Greyson, R. I. (1973) *Can. J. Bot.* **51,** 2473–2479.
17. Jacobsen, S. E. & Olszewski, N. E. (1991) *Plant Physiol.* **97,** 409–414.
18. Kobayashi, M., Yamaguchi, I., Murofushi, N., Ota, Y. & Takahashi, N. (1988) *Agric. Biol. Chem.* **52,** 1189–1194.
19. Kobayashi, M., Kamiya, Y., Sakurai, A., Saka, H. & Takahashi, N. (1990) *Plant Cell Physiol.* **31,** 289–293.
20. Chiang, H.-H., Hwang, I. & Goodman, H. M. (1995) *Plant Cell* **7,** 195–201.
21. Lange, T., Robatzek, S. & Frisse, A. (1997) *Plant Cell* **9,** 1459–1467.
22. Toyomasu, T., Kawaide, H., Mitsuhashi, W., Inoue, Y. & Kamiya, Y. (1998) *Plant Physiol.* **118,** 1517–1523.
23. Yamaguchi, S., Smith, M. W., Brown, R. G. S., Kamiya, Y. & Sun, T.-P. (1998) *Plant Cell*, **10,** 2115–2126.
24. Itoh, H., Ueguchi, M. T., Kawaide, H., Chen, X., Kamiya, Y. & Matsuoka, M. (1999) *Plant J.* **20,** 15–24.
25. Kobayashi, M., Yoshizawa, K., Sakurai, A. & Nakamura, T. (1996) *Biosci. Biotechnol. Biochem.* **60,** 159–160.
26. Murray, M. G. & Thompson, W. F. (1980) *Nucleic Acids Res.* **8,** 4321–4325.
27. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning*: *A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY), 2nd Ed.
28. Hood, E. E., Helmer, G. L., Fraley, R. T. & Chilton, M. D. (1986) *J. Bacteriol.* **168,** 1291–1301.
29. Hiei, Y., Ohta, S., Komari, T. & Kumashiro, T. (1994) *Plant J.* **6,** 271–282.
30. Kouchi, H. & Hata, S. (1993) *Mol. Gen. Genet.* **238,** 106–119.
31. Coles, J. P., Phillips, A. L., Croker, S. J., García-Lepe, R., Lewis, M. J. & Hedden, P. (1999) *Plant J.* **17,** 547–556.
32. Kobayashi, M., Kwak, S.-S., Kamiya, Y., Yamane, H., Takahashi, N. & Sakurai, A. (1991) *Agric. Biol. Chem.* **55,** 249–251.
33. Spray, C. R., Kobayashi, M., Suzuki, Y., Phinney, B. O., Gaskin, P. & MacMillan, J. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 10515–10518.
34. Albone, K. S., Gaskin, P., MacMillan, J., Phinney, B. O. & Willis, C. L. (1990) *Plant Physiol.* **94,** 132–142.
35. Hasegawa, M., Nakajima, M., Takeda, K., Yamaguchi, I. & Murofushi, N. (1995) *Biosci. Biotechnol. Biochem.* **59,** 1716–1720.
36. Smith, V. A., Gaskin, P. & MacMillan, J. (1990) *Plant Physiol.* **94,** 1390–1401.
37. Kinoshita, T. (1995) *Rice Gen. News.* **12,** 9–154.
38. Harushima, Y., Yano, M., Shomura, A., Sato, M., Shimano, T., Kuboki, Y., Yamamoto, T., Lin, S.-Y., Antonio, B. A., Parco, A., *et al*. (1998) *Genetics* **148,** 479–494.
39. Tsunematsu, H., Yoshimura, A., Harushima, Y., Nagamura, Y., Kurata, N., Yano, M., Sasaki, T. & Iwata, N. (1996) *Breeding Sci.* **46,** 279–284.
40. Silverstone, A. L., Chang, C.-W., Krol, E. & Sun, T.-P. (1997) *Plant J.* **12,** 9–19.
41. Hasegawa, M., Hashimoto, N., Zhang, J., Nakajima, M., Takeda, K., Yamaguchi, I. & Murofushi, N. (1995) *Biosci. Biotechnol. Biochem.* **59,** 1925–1929.