An Overview of Gibberellin Metabolism Enzyme Genes and Their Related Mutants in Rice[1][w]

Tomoaki Sakamoto, Koutarou Miura, Hironori Itoh, Tomoko Tatsumi, Miyako Ueguchi-Tanaka, Kanako Ishiyama, Masatomo Kobayashi, Ganesh K. Agrawal, Shin Takeda, Kiyomi Abe, Akio Miyao, Hirohiko Hirochika, Hidemi Kitano, Motoyuki Ashikari, and Makoto Matsuoka*

Field Production Science Center, University of Tokyo, Nishi-Tokyo, Tokyo 188–0002, Japan (T.S.); Bioscience and Biotechnology Center, Nagoya University, Nagoya, Aichi 464–8601, Japan (K.M., H.I., T.T., M.U.-T., H.K., M.A., M.M.); BioResource Center, RIKEN Tsukuba Institute, Tsukuba, Ibaraki 305–0074, Japan (K.I., M.K.); and Molecular Genetics Department, National Institute of Agrobiological Sciences, Tsukuba, Ibaraki 305–8602, Japan (G.K.A., S.T., K.A., A.M., H.H.)

To enhance our understanding of GA metabolism in rice (*Oryza sativa*), we intensively screened and identified 29 candidate genes encoding the following GA metabolic enzymes using all available rice DNA databases: *ent*-copalyl diphosphate synthase (CPS), *ent*-kaurene synthase (KS), *ent*-kaurene oxidase (KO), *ent*-kaurenoic acid oxidase (KAO), GA 20-oxidase (GA20ox), GA 3-oxidase (GA3ox), and GA 2-oxidase (GA2ox). In contrast to the Arabidopsis genome, multiple *CPS*-like, *KS*-like, and *KO*-like genes were identified in the rice genome, most of which are contiguously arranged. We also identified 18 GA-deficient rice mutants at six different loci from rice mutant collections. Based on the mutant and expression analyses, we demonstrated that the enzymes catalyzing the early steps in the GA biosynthetic pathway (i.e. CPS, KS, KO, and KAO) are mainly encoded by single genes, while those for later steps (i.e. GA20ox, GA3ox, and GA2ox) are encoded by gene families. The remaining *CPS*-like, *KS*-like, and *KO*-like genes were likely to be involved in the biosynthesis of diterpene phytoalexins rather than GAs because the expression of two *CPS*-like and three *KS*-like genes (*OsCPS2*, *OsCPS4*, *OsKS4*, *OsKS7*, and *OsKS8*) were increased by UV irradiation, and four of these genes (*OsCPS2*, *OsCPS4*, *OsKS4*, and *OsKS7*) were also induced by an elicitor treatment.

The GAs form a large family of tetracyclic diterpenoid phytohormones that are involved in the regulation of various growth and developmental processes in higher plants. Bioactive GAs, such as GA_1 and GA_4, are synthesized from trans-geranylgeranyl diphosphate (GGDP) as shown in Figure 1 (Hedden and Kamiya, 1997; Hedden and Phillips, 2000). GGDP is converted to the tetracyclic hydrocarbon *ent*-kaurene via *ent*-copalyl diphosphate (CDP) by two kinds of diterpene cyclases in plastids, CDP synthase (CPS) and *ent*-kaurene synthase (KS). *ent*-Kaurene is then modified by sequential oxidations to produce GA_{12} via *ent*-kaurenoic acid. These steps are catalyzed by two membrane-associated Cyt P450 monooxygenases, *ent*-kaurene oxidase (KO) and *ent*-kaurenoic acid oxidase (KAO). The final stage of bioactive GA synthesis, from GA_{53}/GA_{12} to GA_1/GA_4, is catalyzed through two parallel pathways (i.e. early-13-hydroxylation and non-13-hydroxylation pathways) by two soluble 2-oxoglutarate-dependent dioxygenases (2ODDs) in the cytosol, GA 20-oxidase (GA20ox) and GA 3-oxidase (GA3ox). The bioactive GA_1/GA_4 and their immediate precursors GA_{20}/GA_9 are inactivated by a third 2ODD, GA 2-oxidase (GA2ox). In recent years, almost all of the genes encoding these seven GA metabolic enzymes (CPS, KS, KO, KAO, GA20ox, GA3ox, and GA2ox) and their related mutants have been isolated from various plants (Hedden and Phillips, 2000). A typical phenotype of these mutants, except the mutants with GA2ox, is dwarfism, a state that is restored by the application of exogenous GA.

Dwarfism is one of the most valuable traits in crop breeding because semidwarf cultivars are more resistant to damage by wind and rain (lodging resistant) and are associated with stable increased yields (Evans, 1993). Because of their agronomic importance, a large number of dwarf mutants of rice (*Oryza sativa*) have been isolated, and several of them have been characterized as GA-deficient (Murakami, 1972; Kobayashi et al., 1989; Itoh et al., 2001; Sasaki et al., 2002a). In this study, we attempted to identify the genes encoding the seven GA metabolic enzymes and their related mutants to enhance our current understanding of the GA metabolic pathway in rice. Although there is only one *KAO*-like gene in the rice genome, two to nine candidate genes were identified for the other six enzymes.

[1] This work was supported by the Ministry of Agriculture, Forestry and Fisheries of Japan (Rice Genome Project; grant no. IP–1010 to T.S.), the Program for Promotion of Basic Research Activities for Innovation of Biosciences (grant to M.U.-T. and H.K.), and the Ministry of Education, Culture, Sports, Science and Technology (Grant-in-Aid for the Center of Excellence to M.M.).

* Corresponding author; e-mail makoto@nuagr1.agr.nagoya-u.ac.jp; fax 81–52–789–5226.

[w] The online version of this article contains Web-only data.

Article, publication date, and citation information can be found at www.plantphysiol.org/cgi/doi/10.1104/pp.103.033696.

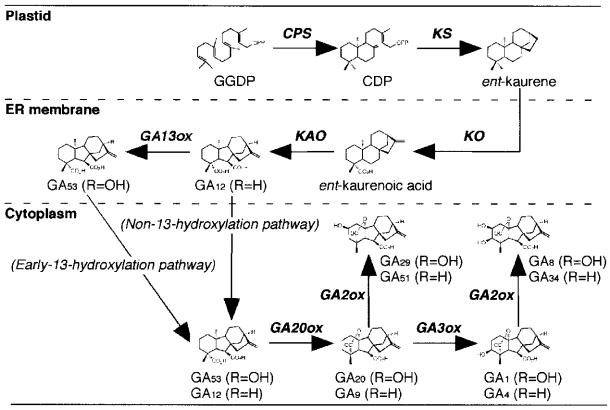

Figure 1. Principal pathway of GA metabolism in plants. GA13ox, GA 13-hydroxylase.

Based on the mutant and expression analyses, we have revealed that the enzymes catalyzing early steps in GA biosynthetic pathway (i.e. CPS, KS, KO, and KAO) are encoded by single genes, while those for later steps (i.e. GA20ox, GA3ox, and GA2ox) are encoded by small gene families. We have also found that some *CPS*-like and *KS*-like genes are likely to be involved in diterpene phytoalexin biosynthesis.

RESULTS

Isolation of Putative GA Metabolic Genes in Rice

The predicted amino acid sequences of GA metabolic enzyme genes from various plants (including Arabidopsis, lettuce [*Lactuca sativa*], pea [*Pisum sativum*], tomato [*Lycopersicon esculentum*], and rice) were used as probes to screen in silico all available rice DNA databases (Feng et al., 2002; Goff et al., 2002; Sasaki et al., 2002b; Yu et al., 2002; Kikuchi et al., 2003). Candidate GA sequences detected during this process were also used reiteratively as search probes. Through this exhaustive sequence search, we found four *CPS*-like genes (*OsCPS1-4*), nine *KS*-like genes (*OsKS1-9*), five *KO*-like genes (*OsKO1-5*), four *GA20ox*-like genes (*OsGA20ox1-4*), two *GA3ox*-like genes (*OsGA3ox1* and *2*), and four *GA2ox*-like genes (*OsGA2ox1-4*), whereas there was only one *KAO*-like gene (*OsKAO*; Table I). Localization of the 29 candidate genes on the rice genome revealed that they are distributed across nine of the 12 rice chromosomes (Fig. 2A). There are five small gene clusters that contain 16 of the 29 candidate genes (shaded in red in Fig. 2A; see below).

CPS-Like Genes

Four *CPS*-like genes were identified in the rice genome, including a pseudogene (*OsCPS3*), the transcript

Table I. *GA biosynthetic genes and their homologs in rice*

Enzyme	Gene Name	Entry Name	Chromosome Location	Comment
CPS	*OsCPS1*	AP004572	Chr. 2, 2.49 cM	GA biosynthesis
	OsCPS2	AP005114	Chr. 2, 86 cM	Linked with *OsKS5*, *OsKS6*, and *OsKS7*
	OsCPS3	AP005767	Chr. 9, 26.7 cM	Pseudogene
	OsCPS4	AL662933	Chr. 4, 14.3 cM	Linked with *OsKS4*
KS	*OsKS1*	OSJN00255	Chr. 4, 104 cM	GA biosynthesis
	OsKS2	OSJN00255	Chr. 4, 104 cM	Linked with *OsKS1* and *OsKS3*
	OsKS3	OSJN00255	Chr. 4, 104 cM	Linked with *OsKS1* and *OsKS2*
	OsKS4	OSJN00145	Chr. 4, 14.3 cM	Linked with *OsCPS4*
	OsKS5	AP005114	Chr. 2, 86 cM	Linked with *OsKS6*, *OsKS7*, and *OsCPS2*
	OsKS6	AP005114	Chr. 2, 86 cM	Linked with *OsKS5*, *OsKS7*, and *OsCPS2*
	OsKS7	AP005114	Chr. 2, 86 cM	Linked with *OsKS5*, *OsKS6*, and *OsCPS2*
	OsKS8	AC135398	Chr. 11, 57.6 cM	Linked with *OsKS9*
	OsKS9	AC135398	Chr. 11, 57.6 cM	Pseudogene
KO	*OsKO1*	AP005471	Chr. 6, 76 cM	Arranged as a gene cluster with 5 tandem repeats
	OsKO2	AP005471	Chr. 6, 76 cM	GA biosynthesis, loss of function induces *d35*
	OsKO3	AP005471	Chr. 6, 76 cM	Arranged as a gene cluster with 5 tandem repeats
	OsKO4	AP005471	Chr. 6, 76 cM	Arranged as a gene cluster with 5 tandem repeats
	OsKO5	AP005471	Chr. 6, 76 cM	Arranged as a gene cluster with 5 tandem repeats
KAO	*OsKAO*	AP000616	Chr. 6, 2 cM	GA biosynthesis
GA20ox	*OsGA20ox1*	AC096690	Chr. 3, 164.6 cM	
	OsGA20ox2	AP003561	Chr. 1, 149.1 cM	Loss of function induces *sd1*
	OsGA20ox3	AP005840	Chr. 7, 26–31 cM	
	OsGA20ox4	AC124836	Chr. 5, 80.4 cM	
GA3ox	*OsGA3ox1*	AC144738	Chr. 5, 33.6 cM	
	OsGA3ox2	AP002523	Chr. 1, 16.4–19.9 cM	Loss-of-function induces *d18*
GA2ox	*OsGA2ox1*	AC119288	Chr. 5, 29.1 cM	
	OsGA2ox2	AP003143	Chr. 1, 67.9 cM	
	OsGA2ox3	AP003375	Chr. 1, 130.1 cM	
	OsGA2ox4	AC132485	Chr. 5, 106 cM	

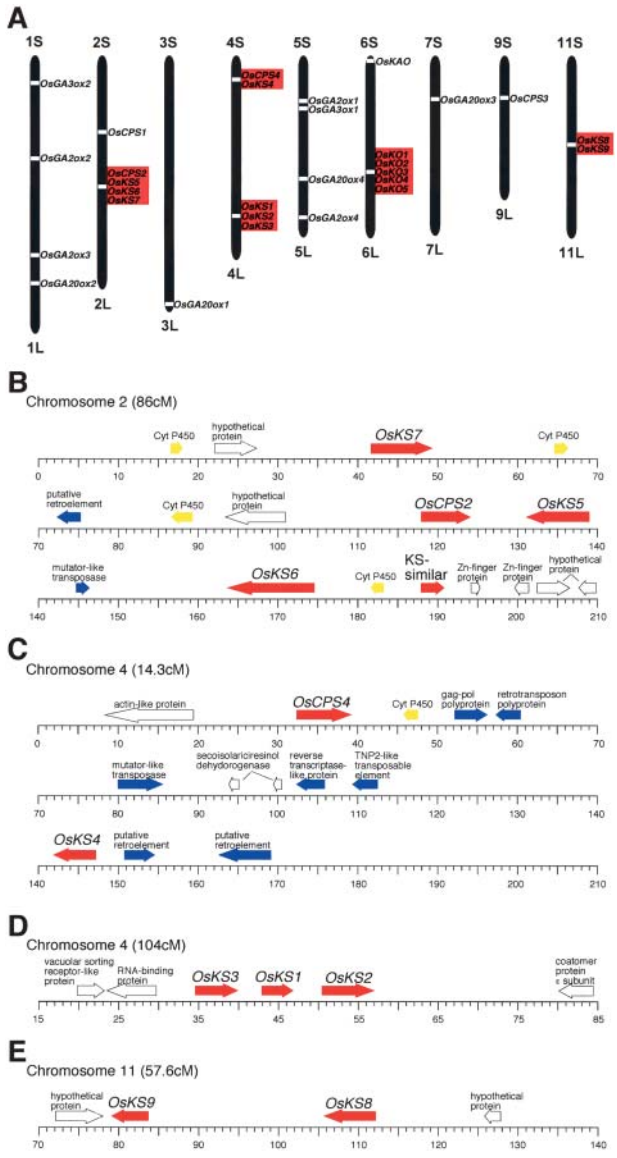

Figure 2. Chromosomal location of the candidate genes for GA metabolism and contiguous arrangement of *CPS*-like and *KS*-like genes. A, Map position of the candidate genes for GA metabolism on the rice genome. B to E, Contiguous arrangement of *CPS*-like and *KS*-like genes. *CPS*-like and *KS*-like genes are indicated by red arrows. Yellow and blue arrows indicate the genes encoding putative Cyt P450 and transposon-like sequences, respectively. White arrows show other predicted genes. The orientation of each gene is indicated by the direction of the arrow. The gene prediction was performed using the RiceGAAS program (http://ricegaas.dna.affrc.go.jp/), alignment with expressed sequence tag sequences, and direct sequence analysis of RT-PCR products. Intergenic regions are drawn to scale and contain no other genes.

of which lacks the coding sequence below exon 8, whereas CPS is encoded by the single gene, *AtCPS/GA1*, in Arabidopsis. Mapping analysis revealed that *OsCPS1* and *OsCPS2* are located on chromosome 2 and that *OsCPS3* and *OsCPS4* are located on chromosomes 9 and 4, respectively (Table I; Fig. 2A). Interestingly, *OsCPS2* on chromosome 2 was contiguously clustered with three *KS*-like genes, *OsKS5*, *OsKS6*, and *OsKS7* (Table I; Fig. 2, A and B). Similarly, *OsCPS4* was located near to another *KS*-like gene, *OsKS4*, on chromosome 4 (Table I; Fig. 2, A and C), whereas *OsCPS3* was mapped onto chromosome 9 without linkage to any *KS*-like genes (Table I; Fig. 2A).

The deduced amino acid sequence of Arabidopsis AtCPS/GA1 showed highest homology with OsCPS1 (41% identity) and relatively lower identities with OsCPS2 to OsCPS4 (ranging between 35% and 37%). An Asp-rich box, DXDDTA, which is shared in many terpene cyclases, catalyzing cyclization without removal of the diphosphate group, was conserved in OsCPS2 (at residues 374–379) and OsCPS4 (at residues 319–324); however, the first Asp was replaced with Glu in OsCPS1 (at residue 418), and the C-terminal half (DTA) was exchanged to TGS in OsCPSL2 (at residues 364–366). A SAYDTAWVA motif that is conserved among CPS and KS proteins was also conserved in OsCPS1 (at residues 155–163) and OsCPS3 (at residues 97–105), whereas amino acid substitutions were observed in OsCPS2 (at residues 109–117) and OsCPS4 (at residues 56–64). Phylogenetic analysis of the cyclases (CPS and KS; Fig. 3A) revealed that OsCPS1 was most closely related to maize CPS, ZmCPS/AN1 (63% identity; Fig. 3A), whereas OsCPS2 to OsCPS4 are grouped as a different clade from the OsCPS1 clade and the CPS proteins in dicot plants. This suggests that cyclases OsCPS2 to OsCPS4 might have different functions from those of OsCPS1 and ZmCPS/AN1 (see below).

KS-Like Genes

Nine *KS*-like genes were identified in the rice genome, including a pseudogene (*OsKS9*), which contained a premature stop codon in exon 3, whereas KS is encoded by the single-copy gene, *AtKS/GA2*, in Arabidopsis. *OsKS1*, *OsKS2*, and *OsKS3* were arranged as tandem direct repeats at 104 centimorgan (cM) of chromosome 4 (Table I; Fig. 2, A and D). *OsKS4* was located near to the position of *OsCPS4* on chromosome 4 as mentioned above (Table I; Fig. 2, A and C). *OsKS5*, *OsKS6*, and *OsKS7* were also arranged within 150 kb at 86 cM of chromosome 2, where *OsCPS2* is also located (Table I; Fig. 2, A and B). *OsKS8* was located on chromosome 11 contiguously to the position of *OsKS9* in the same direction (Table I; Fig. 2, A and E).

OsKS1 showed highest similarity to Arabidopsis AtKS/GA2 (43% identity), whereas the other OsKS proteins showed between 37% and 41% identities with the Arabidopsis protein. The conserved Asp-rich sequence, DDXXD, which is considered to be involved in binding to the diphosphate group of the substrate, was conserved in all OsKS proteins (e.g. at residues 552–556 of OsKS1). In the SAYDTAWVA motif, the first Ala was replaced with Leu in OsKS1 (at residue 80), with Pro in OsKS2, OsKS3, and OsKS7, and with Ser in the other OsKS proteins. The second Ala in the SAYDTAWVA motif was conserved in all OsKS proteins (e.g.

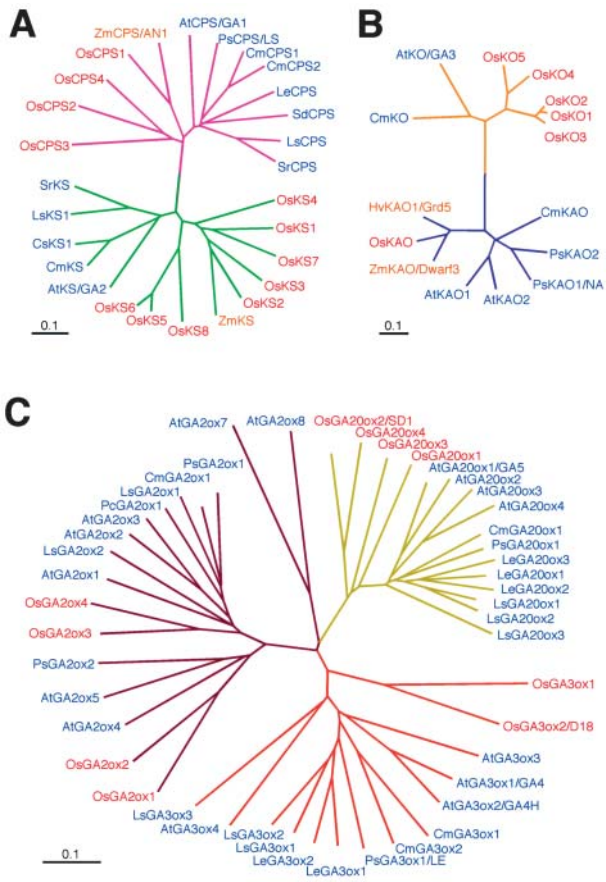

Figure 3. Phylogenetic relationships among GA metabolic diterpene cyclases, CPS and KS (A), GA metabolic Cyt P450-dependent monooxygenases KO and KAO (B), and GA metabolic 2ODDs GA20ox, GA3ox, and GA2ox (C). At, Arabidopsis; Cm, pumpkin; Cs, *Cucumis sativus*; Hv, barley; Le, tomato; Ls, lettuce; Pc, *Phaseolus coccineus*; Ps, pea; Sd, *Scoparia dulcis*; Sr, *Stevia rebaudiana*; and Zm, maize.

at residue 84 of OsKS1), although it was replaced with Ser in AtKS/GA2. Phylogenetic analysis revealed that KS-like proteins are divided into monocot and dicot groups, and the monocot group is subdivided into two further groups. OsKS1-4 and OsKS7 were more closely related to maize (*Zea mays*) ZmKS (49%–54% identities), whereas OsKS5, OsKS6, and OsKS8 constituted another group (39%–43% identities with ZmKS; Fig. 3A).

KO-Like Genes

Five *KO*-like genes were identified in the rice genome, whereas KO is encoded by the single copy gene, *AtKO/GA3*, in Arabidopsis. Mapping analysis of the five *KO*-like genes revealed that they are all mapped to chromosome 6, arranging in tandem as a gene cluster (Table I; Fig. 2A; Itoh et al., 2004). The deduced amino acid sequence of Arabidopsis AtKO/GA3 showed higher identities with OsKO1 to OsKO3 (about 53%) and lower identities with OsKO4 and OsKO5 (47% and 46%). Phylogenetic analysis of GA-related Cyt P450-dependent monooxygenases (Fig. 3B) revealed that OsKO proteins are more similar to each other (64%–93% identities) than to any other reported KO proteins (46%–53% identities) and are subdivided into two groups (i.e. one group contains OsKO1, OsKO2, and OsKO3, and another group involves OsKO4 and OsKO5).

KAO-Like Gene

One *KAO*-like gene was identified in the rice genome, whereas KAO is encoded redundantly by two genes, *AtKAO1* and *AtKAO2*, in Arabidopsis. We searched the databases using several regions of KAO proteins from various plant species, including Arabidopsis, garden pea, pumpkin (*Cucurbita maxima*), barley (*Hordeum vulgare*), and maize, but we could not identify any other genes except *OsKAO*. Mapping analysis revealed that *OsKAO* is located on chromosome 6 (Table I; Fig. 2A). The deduced amino acid sequence of OsKAO showed approximately 51% identities with Arabidopsis AtKAO1 and AtKAO2. Phylogenetic analysis revealed that KAO-like proteins from monocot plants share higher amino acid identities with each other (76%–77% identities) and lower identities with those of dicot plants (51%–54% identities; Fig. 3B). OsKAO is categorized into the monocot group, which includes maize ZmKAO/Dwarf3 (77% identity) and barley HvKAO1/Grd5 (76% identity).

GA20ox-Like Genes

Four *GA20ox*-like genes were identified in the rice genome, two of which (*OsGA20ox1* and *OsGA20ox2*) were identical to the genes reported previously (Toyomasu et al., 1997; Sasaki et al., 2002a); in comparison, five copies of *GA20ox* genes have been reported in Arabidopsis (Hedden et al., 2002). Mapping analysis revealed that *OsGA20ox1* is mapped to chromosome 3 (Table I; Fig. 2A). *OsGA20ox2* is located on the long arm of chromosome 1, which is identical to the rice Green Revolution gene, *Semi-Dwarf1* (*SD1*; Table I; Fig. 2A; Sasaki et al., 2002a). *OsGA20ox3* and *OsGA20ox4* were mapped to chromosomes 7 and 5, respectively (Table I; Fig. 2A).

The deduced amino acid sequence of OsGA20ox1 showed highest homology with Arabidopsis AtGA20-ox1/GA5 (51% identity), whereas OsGA20ox2 to OsGA20ox4 showed 45% to 48% identities to it, respectively. The consensus sequence NYYPXCXXP of 2ODDs for binding the common cosubstrate, 2-oxoglutarate (e.g. at residues 216–224 of OsGA20ox1) and the three His residues (e.g. at residues 79, 233, and 289 of OsGA20ox1) for binding Fe^{2+} were conserved in all OsGA20ox proteins. A sequence LPWKET, which is considered to be involved in the binding of the GA substrates, was also conserved in all OsGA20ox proteins (e.g. at residues 128–133 of OsGA20ox1). Phylogenetic analysis of 2ODDs (GA20ox, GA3ox, and GA2ox; Fig. 3C) revealed that the GA20ox proteins

from dicot plants shared higher amino acid identity each other (49%–80% identities) and formed a single group. OsGA20ox2 showed higher similarity (61% identity) to OsGA20ox4 than the other OsGA20ox proteins, and OsGA20ox2 and OsGA20ox4 formed one subgroup (36%–48% identities with dicot proteins), whereas OsGA20ox1 and OsGA20ox3 were separately located from the OsGA20ox2/OsGA20ox4 subgroup (39%–54% and 39%–49% identities with dicot proteins, respectively).

GA3ox-Like Genes

Two *GA3ox*-like genes were identified in the rice genome, both of which have been already reported by Itoh et al. (2001), whereas four copies of *GA3ox* genes were reported in Arabidopsis (Hedden et al., 2002). Mapping analysis revealed that *OsGA3ox1* is located on the short arm of chromosome 5 (Table I; Fig. 2A), and *OsGA3ox2* is located at the short arm of chromosome 1, which is identical to the rice dwarf gene, *D18* (Table I; Fig. 2A; Itoh et al., 2001).

The deduced amino acid sequence of OsGA3ox1 and OsGA3ox2 showed 29% and 33% identities with Arabidopsis AtGA3ox1/GA4, respectively. Two His residues (e.g. at residues 245 and 303 of OsGA3ox1) and one Asp residue (e.g. at residue 247 of OsGA3ox1) at cofactor binding site were conserved in OsGA3ox1 and OsGA3ox2. The phylogenetic analysis revealed that OsGA3ox1 and OsGA3ox2 are more similar to each other (51% identity) than to any other reported GA3ox proteins from dicot plants (27%–35% identities) and constituted a separate group (Fig. 3C). As there are no reports describing the structure of *GA3ox* genes in monocot plants except rice at present, we have to wait to conclude that the rice *GA3ox* genes show higher similarity to other genes from monocot plants than those from dicots.

GA2ox-Like Genes

Four *GA2ox*-like genes were identified in the rice genome, three of which (*OsGA2ox1*, *OsGA2ox2*, and *OsGA2ox3*) were identical to the genes reported previously (Sakamoto et al., 2001; Sakai et al., 2003), whereas seven copies have been previously reported in Arabidopsis (Schomburg et al., 2003). Mapping analysis revealed that *OsGA2ox1* and *OsGA2ox4* are located on chromosome 5, and *OsGA2ox2* and *OsGA2ox3* are mapped on chromosome 1 (Table I; Fig. 2A). In contrast to the genes encoding the early steps in GA biosynthesis, such as the *CPS*-like, *KS*-like, and *KO*-like genes, *OsGA2ox* genes located on the same chromosomes do not form gene clusters but instead are separated by large distances.

Two His residues (e.g. at residues 241 and 302 of OsGA2ox1) and one Asp residue (e.g. at residue 243 of OsGA2ox1), which are supposed to associate at the catalytic site and to bind with Fe^{2+}, respectively, were conserved in all the rice GA2ox proteins that we found. Phylogenetic analysis revealed that GA2ox proteins are subdivided into three groups. Two Arabidopsis GA2ox proteins, AtGA2ox7 and AtGA2ox8, constituted a separate branch that showed less similarity to the other GA2ox proteins (16%–23% identities with the others). The remaining two groups contained both monocot and dicot plant proteins (i.e. OsGA2ox1 and OsGA2ox2 were grouped with AtGA2ox4, AtGA2ox5, and PsGA2ox2, whereas OsGA2ox3 and OsGA2ox4 were grouped with AtGA2ox1, AtGA2ox2, AtGA2ox3, and the others; Fig. 3C). The arrangement of these three subgroups suggests that these related subgroups of GA2ox proteins might have homologous functions crossing between monocot and dicot plants (Sakai et al., 2003; Schomburg et al., 2003).

Isolation of Rice GA-Deficient Mutants

In silico screening of GA metabolic enzyme genes in rice DNA databases revealed that rice contains 29 candidate genes related to GA metabolism. However, this does not mean that all of these candidate genes are involved in GA metabolism, and it is highly likely that some of them are not related to GA but to other metabolisms. To elucidate which candidate genes are involved in GA biosynthesis, we took a genetic approach; that is, when the genes involved in GA biosynthesis are deficient in their function, mutant plants should exhibit a GA-deficient phenotype. Using this method, we performed a large-scale screening of rice GA-deficient mutants to identify the genes involved in GA biosynthesis.

Figure 4A shows a typical phenotype of a rice GA-deficient mutant at the young seedling stage. The GA-related mutants showed dwarfism without the induction of additionally aberrant morphology. The final plant height of GA-deficient mutants ranged widely between <5% and 90% of the wild-type plants. The leaf blades of the mutant plants became dark green, shorter, and wider than those of the wild-type plants. In contrast to GA-insensitive mutants, exogenously applied bioactive GA_3 rescued the dwarfism of GA-deficient mutants to restore final plant heights similar to those of the wild type (Fig. 4B).

Based on these phenotypic features, we performed a large-scale screening of rice mutant collections, which were produced by a chemical mutagen, a retrotransposon (*Tos17*), and γ-ray irradiation. Through the first screening of these mutant collections, we selected more than several hundred dwarf mutants as candidates. We analyzed the detailed phenotype and GA responsibility of these candidates and finally identified 18 lines as GA-deficient mutants (Table II; Supplemental Fig. 1). Among them, nine lines were derived from the traditional GA-deficient mutants (four alleles of *sd1*, four alleles of *d18*, and one allele of *d35*; Ogawa et al., 1996; Itoh et al., 2001; Ashikari et al., 2002; Sasaki et al., 2002a), and the remaining nine mutant lines with recessive alleles were novel mutants. All 18 mutants could be categorized into three groups by their phenotypes; namely, severe dwarf without flower or

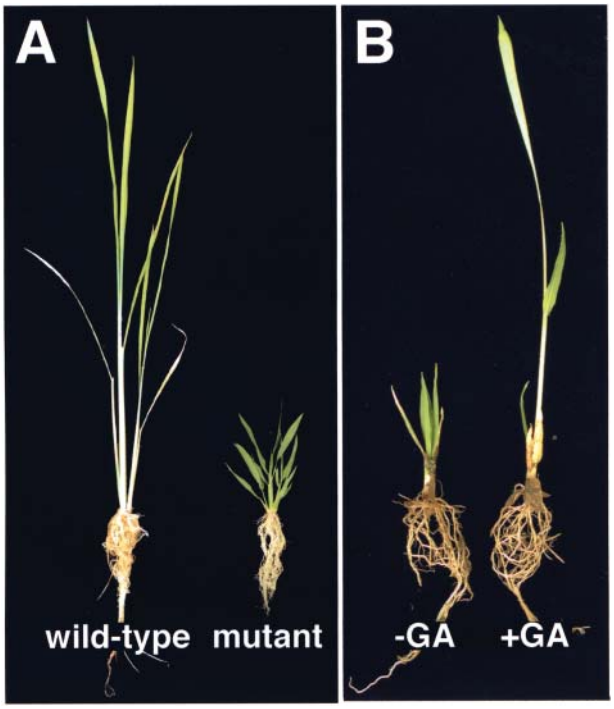

Figure 4. Typical phenotype of GA-deficient rice dwarf mutants (A) and rescue of the dwarf phenotype by external GA_3 treatment (B). We used three criteria for screening of the GA-deficient mutants, that is, dwarfism without aberrant morphology, dark green leaves, and restoration of dwarfism to the wild type by the GA_3 treatment.

seed development (category I; Fig. 5, B–E; Supplemental Fig. 2), severe dwarf with seed development (category II; Fig. 5F), and semidwarf with seed development (category III; Fig. 5A, plants 5, 9, and 11). Seeds of category II and category III mutants were normally germinated (data not shown).

Mapping analysis revealed that each of the nine novel dwarf mutants could be assigned to mutation sites at one of four loci. The mutation sites of two lines, *oscps1-1* (Fig. 5, A, plant 2, and B) and *oscps1-2*, both of which showed severe dwarf phenotype without flower or seed development (category I), were mapped onto chromosome 2, tightly linked to *OsCPS1*. The sequence of *OsCPS1* in these two lines revealed that *oscps1-1* had an insertion of *Tos17* in exon 7, whereas *OsCPS1* in *oscps1-2* had nine nucleotides deleted in exon 7, which encode the well-conserved three amino acid sequence, DLF (Fig. 6). No bioactive GA_1 was detected in *oscps1-1* (Table III). In addition, the level of *ent*-kaurene was severely decreased in *oscps1-1* compared to its original strain, Nipponbare (Table III). This result supports the hypothesis postulated above that *oscps1* had a defect in the activity of CPS, which catalyzes the formation of CDP, the immediate precursors of *ent*-kaurene.

The mutation sites of three other lines, *osks1-1* (Fig. 5, A, plant 3, and C), *osks1-2*, and *osks1-3*, all of which showed severe dwarf phenotype without flower or seed development (category I), were mapped onto chromosome 4, tightly linked to the gene cluster of *OsKS1* to *OsKS3*. Sequence analysis of the *OsKS* genes in these three mutant lines revealed that *OsKS1* was deleted approximately 4.5 kb nucleotides, including the C-terminal half of OsKS1 protein in *osks1-3*, whereas *osks1-1* and *osks1-2* had a *Tos17* insertion in exons 3 and 14 of *OsKS1*, respectively (Fig. 6). No mutation was observed in the *OsKS2* or *OsKS3* gene from the three mutant lines. Again, no bioactive GA_1 was detected in *osks1-1* (Table III). Similar to the case of *oscps-1*, the level of *ent*-kaurene was severely decreased in *osks1-1* compared to its original strain, Nipponbare (Table III). The result is consistent with the hypothesis that *osks1* had a defect in the activity of KS, which catalyzes the formation of *ent*-kaurene from CDP.

The mutation site of *osko2-1* (Fig. 5, A, plant 6, and D), which showed severe dwarf phenotype without flower or seed development (category I), was mapped onto chromosome 6, tightly linked to the cluster of *OsKO1* to *OsKO5*. Sequence analysis of the five *OsKO*

Table II. *The original mutant names, their wild-type parental lines, and the mutagen used*

Gene	Allele Name	Original Name	WT Parent Line	Mutagen
OsCPS1	*oscps1-1*	NE3024	Nipponbare	*Tos17*
	oscps1-2	K2540	Nipponbare	Spontaneous
OsKS1	*osks1-1*	ND4002	Nipponbare	*Tos17*
	osks1-2	NG5080	Nipponbare	*Tos17*
	osks1-3	F5182	Nipponbare	Spontaneous
OsKO2	*osko2-1*	NE8519	Nipponbare	*Tos17*
	osko2-2	*d35*/Tanginbozu	Ginbozu	Spontaneous
OsKAO	*oskao-1*	NC2581	Nipponbare	*Tos17*
	oskao-2	F6751	Nipponbare	Spontaneous
	oskao-3	NC7607	Nipponbare	*Tos17*
OsGA20ox2	*sd1-1*	Dee-geo-woo-gen	Woo-gen	Spontaneous
	sd1-2	Jikkoku	Unknown	Spontaneous
	sd1-3	Calrose76	Calrose	γ-ray
	sd1-4	Reimei	Fujiminori	γ-ray
OsGA3ox2	*d18-AD*	Akibare-waisei	Akibare	Spontaneous
	d18-dy	Waito-C	Unknown	Spontaneous
	d18k	Kotaketamanishiki	Tamanishiki	Spontaneous
	d18h	Housetsu-waisei	Unknown	Spontaneous

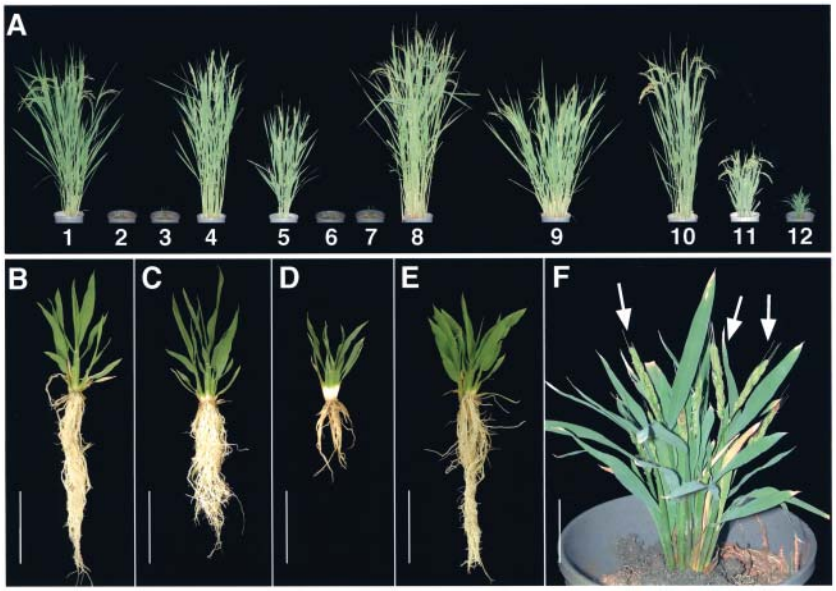

Figure 5. Phenotypes of rice GA-deficient dwarf mutants. A, Comparison of gross morphology between mutant plants and their original cultivars. 1, Nipponbare (original strain for *oscps-1*, *osks1-1*, *osko2-1*, and *oskao-1*); 2, *oscps-1* (null allele); 3, *osks1-1* (null allele); 4, Ginbozu (original strain for *osko2-2*); 5, *osko2-2* (weak allele); 6, *osko2-1* (null allele); 7, *oskao-1* (null allele); 8, Woo-gen (original strain for *sd1-1*); 9, *sd1-1* (null allele); 10, Akibare (original strain for *d18-AD*); 11, *d18-dy* (weak allele); and 12, *d18-AD* (null allele). B to F, Close-up view of *oscps-1*, *osks1-1*, *osko2-1*, *oskao-1*, and *d18-AD*, respectively. Arrows indicate panicles containing fertile seeds. Bar represents 5 cm.

genes in *osko2-1* revealed that *Tos17* was inserted into exon 4 of *OsKO2* (Fig. 6). Recently, we have also demonstrated that the *d35* mutant (Fig. 5A, plant 5), which showed semidwarf phenotype with seed development (category II), is due to a loss of function of the *OsKO2* gene (T. Tatsumi, H. Itoh, and M. Matsuoka, unpublished data). The *OsKO2* sequence in *d35* had a single nucleotide substitution in exon 5, which induced an amino acid change (Fig. 6); therefore, we designated *d35*/Tanginbozu as *osko2-2*, a weak allele of *OsKO2*. The observed levels of GA_{53}, GA_{44}, GA_{19}, GA_{20}, and GA_1 were lower in *osko2-2* than in the original strain, Ginbozu, whereas *ent*-kaurene was highly accumulated (approximately 10-fold) in *osko2-2* (Table III). This result supports the hypothesis that *osko2* is deficient in the KO activity, which catalyzes the formation of *ent*-kaurenoic acid from *ent*-kaurene, as has already been suggested (Ogawa et al., 1996).

The mutation sites of the final three lines, *oskao-1* (Fig. 5, A, plant 7, and E), *oskao-2*, and *oskao-3*, which showed severe dwarf phenotype without flower or seed development (category I), were mapped onto chromosome 6, tightly linked to *OsKAO*. Sequence analysis of OsKAO from these three lines revealed that *oskao-2* had a single nucleotide substitution in exon 1, which induced an exchange of highly conserved amino acid residue among this kind of Cyt P450 monooxygenases, whereas the other two lines (*oskao-1* and *oskao-3*) had a *Tos17* insertion in exons 5 and 6 of *OsKAO*, respectively (Fig. 6). The levels of GA_{53}, GA_{44}, GA_{19}, GA_{20}, and GA_1 in *oskao-1* were severely decreased relative to those in its original strain, Nipponbare (Table III). The level of *ent*-kaurene in *oskao-1* was less decreased than that in *oscps-1* and *osks1-1*. The result implies that *oskao* had a severe defect not in the formation of *ent*-kaurene but in the activity of KAO, which catalyzes the formation of GA_{12}, the precursors of GA_{53}.

Figure 5 also shows the traditional GA-deficient mutants such as *sd1* and *d18*. All four *sd1* mutant alleles showed a semidwarf phenotype, which is favorable for the breeding of high-yielding cultivars

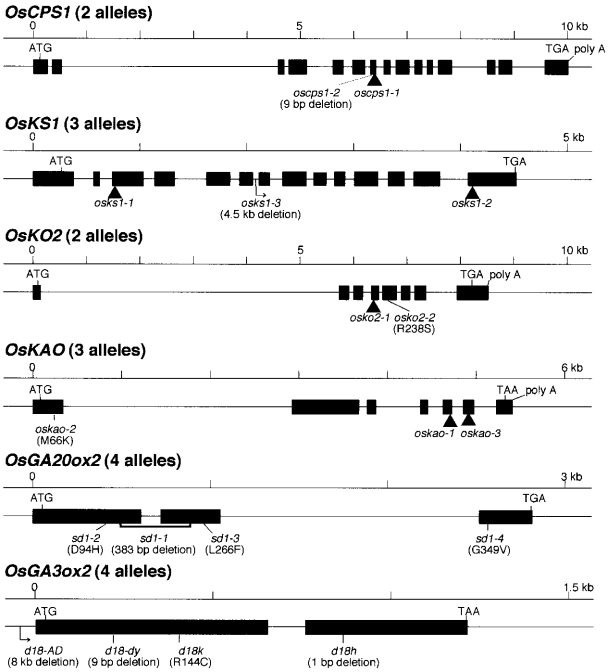

Figure 6. Genomic structure of the GA metabolic genes. Boxes and lines indicate exons and introns, respectively. The mutation sites are indicated below each line. Arrowheads indicate the insertion sites of a rice retrotransposon, *Tos17*. Nucleotide substitutions producing amino acid exchanges are indicated by X numeral Y (X, amino acid residue in the wild-type allele; numeral, the position of exchanged amino acid residue; Y, amino acid residue in the mutant allele).

Table III. *Endogenous levels (ng g^{-1} fresh weight) of various GAs in wild-type and mutant seedlings*

	ent-Kaurene	GA_{53}	GA_{44}	GA_{19}	GA_{20}	GA_1	GA_{29}	GA_8
Nipponbare (WT)	9.60	0.92	0.21	13.0	0.19	0.24	NA[a]	NA
oscps-1	ND[b,c]	0.28	ND	ND	0.15	ND	NA	NA
osks1-1	ND[c]	0.29	0.19	ND	0.15	ND	NA	NA
oskao-1	5.25[c]	0.19	ND	ND	0.05	ND	NA	NA
Ginbozu (WT)	4.40	1.10	0.65	24.0	0.22	0.31	NA	NA
osko2-2	45.0	0.96	0.14	0.89	0.17	0.21	NA	NA
Usen (WT)	NA	0.53	0.29	3.80	0.19	0.25	0.55	0.93
sd1-1	NA	0.62	0.19	3.20	0.11	0.05	0.28	0.35
Akibare (WT)	NA	1.00[c]	0.23[c]	10.9[c]	0.13[c]	0.04	0.23	0.29
d18-AD	NA	0.73[c]	0.52[c]	5.60[c]	1.25[c]	ND	1.00	ND

[a]NA, Not analyzed. [b]ND, Not detected (*ent*-kaurene, <1 ng g^{-1} fresh weight; GAs, <0.02 ng g^{-1} fresh weight). [c]Numbers are the average of two separate measurements (see Supplemental Table I).

(category III; Fig. 5A, plant 9; Ashikari et al., 2002). In *d18* mutants, two lines, *d18-dy* (Fig. 5A, plant 11) and *d18k*, exhibited a semidwarf phenotype (category III) caused by weak alleles, whereas the remaining two lines, *d18-AD* (Fig. 5, A, plant 12, and F) and *d18h*, were severely dwarfed (category II) by the null alleles (Itoh et al., 2001). It is noteworthy that reproductive development was not abolished even in the null alleles of *d18* (arrows in Fig. 5F), although the mutant's vegetative development was severely affected. In contrast to these traditional mutant lines, both vegetative and reproductive developments were severely restricted in the novel GA-deficient mutants as mentioned above (Fig. 5, B–E), probably because rice geneticists and breeders historically screened only self-fertile mutants.

As already reported (Ashikari et al., 2002; Sasaki et al., 2002a), *sd1-1* was caused by a 383-bp deletion, which induced a frameshift and created a premature stop codon (Fig. 5, plant 9), whereas the other three *sd1* alleles had single nucleotide substitutions, which induced amino acid changes (*sd1-2*, *sd1-3*, and *sd1-4*; Fig. 6). The levels of GA_{44}, GA_{19}, GA_{20}, GA_1, GA_{29}, and GA_8 in *sd1-1* were lower than in the original strain, Usen, whereas the amount of GA_{53} in *sd1-1* was slightly higher (Table III). This result confirms that the activity of GA20ox, which catalyzes the steps from GA_{53} to GA_{20} via GA_{44} and GA_{19}, was weaker in *sd1* than in the wild-type plants.

In the strong alleles of the *d18* mutants, *d18-AD* (Fig. 5F) showed an 8-kb deletion that includes the entire coding sequence of *OsGA3ox2* (Fig. 6). In another strong allele, *d18h*, a 1-bp deletion in exon 2 shifts the reading frame, as reported by Itoh et al. (2001; Fig. 6). In the weak alleles, *d18-dy* showed a 9-bp deletion in exon 1, which induced a 3-amino acid deletion, and *d18k* had a single nucleotide substitution in exon 1, which induced an amino acid change (Fig. 6). Although no bioactive GA_1 was detected in *d18-AD*, GA_{20} accumulated at an approximately 10-fold increase over its original strain, Akibare, at the vegetative stage (Table III). This result strongly suggests that *d18-AD* is defective in the activity of GA3ox, which catalyzes the step from GA_{20} to GA_1.

Expression of GA Metabolic Genes in Wild-Type Rice

Semiquantitative reverse transcription (RT)-PCR analysis revealed that the isolated GA metabolic genes were expressed at different levels in various organs of wild-type rice (Fig. 7). *OsCPS1*, *OsKS1*, *OsKO2/D35*, and *OsKAO* were broadly expressed in all the organs of wild-type rice that we tested, including vegetative shoot apices, leaf sheaths, leaf blades, elongating stems, roots, immature panicles, and panicles at flowering time, whereas the levels of *OsKS1* in roots and of *OsKAO* in leaf sheathes and stems were lower than those in other organs.

All *OsGA20ox* genes were expressed in immature and mature panicles at different levels. In addition,

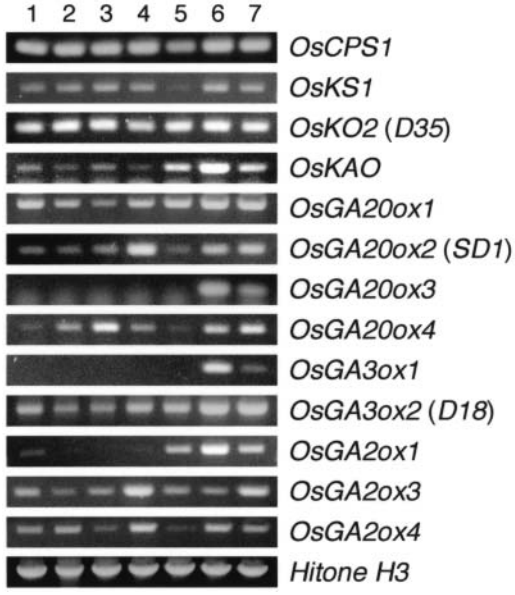

Figure 7. Expression of the GA metabolic genes in various organs of the wild-type rice. Total RNAs were isolated from vegetative shoot apices (1), leaf sheaths (2), leaf blades (3), stems (4), roots (5), immature panicles (6), and panicles at flowering time (7), and quantitative RT-PCR was conducted (see "Materials and Methods"). Histone H3 was used as a control.

OsGA20ox1 was expressed in all vegetative organs that we tested. *OsGA20ox2/SD1* transcript was highly accumulated in stems and also in other vegetative organs. *OsGA20ox3* expression was not observed in any vegetative organs, whereas *OsGA20ox4* was moderately expressed in leaf sheaths, leaf blades, and stems and weakly in vegetative shoot apices and roots. The expression profile suggests that OsGA20ox2/SD1 is the dominant GA20ox in stems and that *OsGA20ox1* and *OsGA20ox4* could be also involved in GA biosynthesis in vegetative organs. This hypothesis corresponds well to the weak phenotype of the *sd1* mutant relative to other GA-deficient rice mutants. Similarly to the case of *OsGA20ox3*, *OsGA3ox1* was preferentially expressed in the panicles. In contrast, *OsGA3ox2* was broadly expressed in all of the organs that we tested, corresponding to the severe phenotype of *d18-AD* at the vegetative stage but the mild phenotype at the reproductive stage.

OsGA2ox1 was preferentially expressed in roots, immature panicles, and mature panicles and also in vegetative shoot apices at low level. Transcripts were not observed in leaf sheaths, leaf blades, or stems, consistent with earlier studies (Sakamoto et al., 2001), although faint signals were detected in these organs when the cycle number of the PCR was increased (data not shown). Both *OsGA2ox3* and *OsGA2ox4* were broadly expressed in all organs of the wild-type rice we tested, whereas *OsGA2ox2* expression was not detected in any organs (data not shown).

Although *CPS*-like, *KS*-like, and *KO*-like genes constitute small gene families, loss-of-function mutants of *OsCPS1*, *OsKS1*, and *OsKO2* showed severe defects in both vegetative and reproductive development (category I). These results strongly suggest that only one gene for each enzyme functions for bioactive GA synthesis in rice. Because rice produces diterpene phytoalexins that are considered to be synthesized from GGDP (Wickham and West, 1992; Mohan et al., 1996), it is possible that some *CPS*-like and *KS*-like genes are involved in the biosynthesis of phytoalexins rather than GAs. Phytoalexin production is stimulated by various kinds of inducers, such as UV irradiation and the elicitors produced by pathogenic microorganisms (Wickham and West, 1992; Kato et al., 1995). We therefore examined *CPS*-like and *KS*-like gene expression in UV-irradiated rice seedlings and elicitor-treated rice cell cultures.

The expression of *OsCPS1* and *OsKS1*, which function for GA biosynthesis, was unaltered in UV irradiated rice seedlings (Fig. 8). Similarly, *OsKS3*, *OsKS5*, and *OsKS6* expression was not affected by the UV irradiation. On the other hand, the expression of *OsCPS2*, *OsCPS4*, *OsKS4*, *OsKS7*, and *OsKS8* was greater in UV irradiated seedlings in comparison to the nonirradiated seedlings (Fig. 8). *OsKS2* expression was not detected either in the nontreated or treated seedlings (data not shown).

In the cultured cells, transcript of *OsCPS1*, *OsKS1*, *OsKS2*, *OsKS6*, or *OsKS8* was not detected in either the elicitor-treated or nontreated conditions (data not shown). Similarly to the case of UV-irradiated seedlings, *OsKS3* or *OsKS5* expression rate was not affected by the elicitor treatment. On the other hand, the expression of *OsCPS2*, *OsCPS4*, *OsKS4*, and *OsKS7* was increased in the elicitor-treated cells compared to the nontreated cells (Fig. 8). The level of *OsCPS2*, *OsCPS4*, and *OsKS7* transcripts gradually increased for 12 h after the elicitor treatment. The *OsKS4* transcript increased until 6 h after the treatment, following which the expression level decreased.

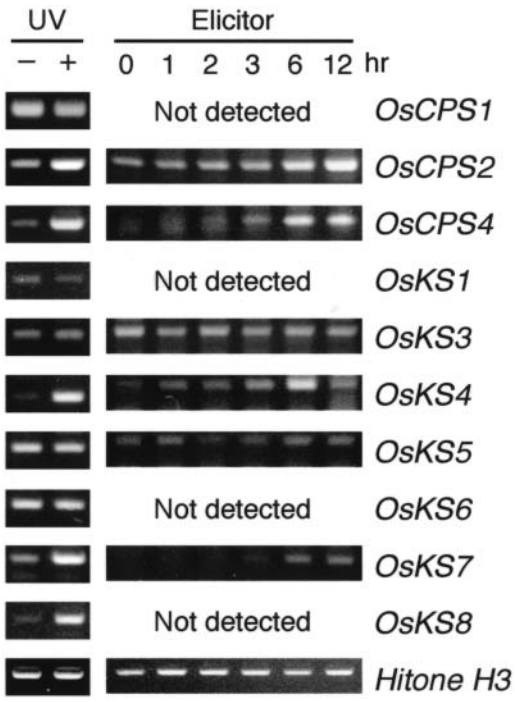

Figure 8. Effect of UV irradiation and elicitor treatment on the levels of *CPS*-like and *KS*-like gene expression. Left, Comparison between UV irradiated for 30 min (+) and nonirradiated (−) wild-type seedlings. Right, Cultured cells of wild-type rice were harvested at 0, 1, 2, 3, 6, and 12 h after the elicitor treatment. Experiments were performed as in Figure 7. No PCR product was detected from RNA isolated from cultured cells using the combination of primers for *OsCPS1*, *OsKS1*, *OsKS6*, or *OsKS8*. Histone H3 was used as a control.

DISCUSSION

In silico screening of the rice genomic and cDNA databases identified 29 candidate genes for seven GA metabolic enzymes. Four *CPS*-like, nine *KS*-like, and five *KO*-like genes were identified in the rice genome (Table I), whereas the Arabidopsis genome contains only one gene encoding each of CPS, KS, or KO (Hedden and Phillips, 2000). Interestingly, 11 genes out of 13 *CPS*-like and *KS*-like genes are arranged into four clusters on the rice chromosomes (Fig. 2, B–E). Similarly, the rice genome contains several *GA20ox*-like, *GA3ox*-like, and *GA2ox*-like genes, but their

arrangement differs from that of the *OsCPS*, *OsKS*, and *OsKO* genes, and each gene is arranged separately on the genome. We could not find any sequences homologous to the reported *KAO* genes except *OsKAO*, and the mutation of *OsKAO* induced the severely dwarfed phenotype, indicating that KAO is encoded by a single gene, in contrast to other GA-metabolic enzymes.

To identify which gene(s) is actually involved in GA biosynthesis, we have collected and characterized 18 GA-deficient dwarf mutants. We categorized these mutants into three groups according to their phenotypes, that is, severe dwarf without flower or seed development (category I), severe dwarf with seed development (category II), and semidwarf suitable for breeding of high-yielding cultivars (category III). In the mutants grouped into the category I, bioactive GA production at the vegetative and reproductive stages was severely restricted, suggesting that the mutated gene encodes major enzymes involved in GA biosynthesis at both the vegetative and reproductive stages. In this context, the major enzymes involved in the early steps of GA biosynthesis (CPS, KS, KO, and KAO) are likely to be encoded by single genes (*OsCPS1*, *OsKS1*, *OsKO2*, and *OsKAO*, respectively) that are broadly expressed in both the vegetative and reproductive organs of the wild-type rice because the loss of function of these genes (three alleles of *oscps1*, two alleles of *osks1*, one allele of *osko2*, and three alleles of *oskao*) caused the severe phenotype characterized in the category I.

The null alleles of the loss-of-function mutants of *OsGA3ox2*, *d18-AD*, and *d18h*, were grouped into the category II. The phenotype suggests that bioactive GA production for shoot elongation is severely defective but that for reproductive development is less affected. This indicates that one gene (*OsGA3ox2*) contributes to the GA biosynthesis in shoot elongation, whereas another gene (*OsGA3ox1*) functions redundantly in the reproductive organ development. Supporting this hypothesis, both OsGA3ox1 and OsGA3ox2 produced in *Escherichia coli* showed GA3ox activity in vitro (Itoh et al., 2001), and *OsGA3ox2* was broadly expressed in both vegetative and reproductive organs, whereas *OsGA3ox1* was specifically expressed in the reproductive organs of wild-type rice (Fig. 7).

Functional redundancy in GA20ox proteins explains why the null mutation in *OsGA20ox2*, *sd1*, exhibited a semidwarf phenotype (category III). In addition to *OsGA20ox2*, other *GA20ox* genes, *OsGA20ox1* and *OsGA20ox4*, were simultaneously expressed in all vegetative organs of rice, and all *OsGA20ox* genes were expressed in the reproductive organs. This overlap expression pattern, accompanied with the feedback up-regulation of other GA biosynthetic enzymes by the homeostatic system (Hedden and Phillips, 2000), compensate the defect in OsGA20ox2 function in shoot elongation, and consequently the defect in *OsGA20ox2/SD1* induces suitable semidwarfism of the rice height for useful breeding.

GA2ox genes were also encoded by a small gene family in rice, and two of the four *OsGA2ox* genes (*OsGA2ox3* and *OsGA2ox4*) were broadly expressed in all organs that we tested. When the *GA2ox* genes involved in GA catabolism are deficient in their function, mutant plants should show an elongated slender phenotype, as observed in the *sln* mutant of garden pea (Lester et al., 1999; Martin et al., 1999). We performed a large-scale screening of rice slender mutants and characterized more than 10 lines. However, all these mutants were allelic to the constitutive GA-responsive mutant, *slender rice1* (Ikeda et al., 2001), and we could not identify the loss-of-function mutants for any of the *OsGA2ox* genes (data not shown). These results suggest that at least two GA2ox proteins, OsGA2ox3 and OsGA2ox4, which were simultaneously expressed in all organs of wild-type rice, function redundantly in the control of bioactive GA levels.

Although several *CPS*-like and *KS*-like genes were identified in rice, GA biosynthesis depends on only *OsCPS1* and *OsKS1*. This begs the question of what functions do the other genes homologous to *CPS* and *KS* genes possess? The two successive type B and type A cyclizations, which are catalyzed by CPS and KS, respectively, are shared by the two pathways for GA biosynthesis and for the biosynthesis of polycyclic diterpenes, including rice diterpene phytoalexins, such as oryzalexins and momilactones (Mohan et al., 1996). Therefore, we suspected that some of the *CPS*-like and *KS*-like genes might encode enzymes involved in phytoalexin biosynthesis. As expected, the expression of two *CPS*-like (*OsCPS2* and *OsCPS4*) and three *KS*-like (*OsKS4*, *OsKS7*, and *OsKS8*) genes were increased by UV irradiation, and four of these genes, *OsCPS2*, *OsCPS4*, *OsKS4*, and *OsKS7*, were also induced by the elicitor treatment. Therefore, these *CPS*-like and *KS*-like genes are considered likely to be involved in diterpene phytoalexin biosynthesis in response to pathogen infection and UV irradiation. This hypothesis is supported by the recent characterization of a rice diterpene cyclase gene, *OsDTC1*, which is identical to *OsKS7*. Biochemical studies revealed that OsDTC1 functions as *ent*-cassa-12,15-diene synthase, which is considered to play a key role in the biosynthesis of diterpenoid phytoalexins, (-)-phytocassanes (Cho et al., 2004). The other group has also determined the in vitro activity of OsCPSs and found that some OsCPSs are likely to be involved in the phytoalexin biosynthesis (T. Toyomasu, personal communication).

It is noteworthy that these UV and elicitor inducible *CPS*-like and *KS*-like genes are contiguously arranged at the same loci of chromosomes 2, 4, and 11 (Fig. 2, B–E). Furthermore, the regions at 86 cM of chromosome 2 (one *CPS*-like gene, three *KS*-like genes, and one *KS*-homologous sequence) and at 14.3 cM of chromosome 4 (one *CPS*-like gene and one *KS*-like gene) also contain genes encoding putative Cyt P450s, which are similar to geraniol 10-hydroxylase (Fig. 2, B and C). Such a contiguous arrangement of genes involved in

consecutive steps in terpenoid biosynthesis also occurs in the Arabidopsis genome (Aubourg et al., 2002). Aubourg et al. (2002) predicted that the biological meaning of the clustered organization of terpenoid synthase (TPS) genes for consecutive steps is for coordinated gene expression in the multiply stepped modules of terpenoid biosynthesis. According to their prediction, the *CPS*-like and *KS*-like genes clustered in these loci might be coordinately regulated by the UV irradiation and elicitor treatments; however, in the Arabidopsis genome, the gene clusters do not consist of TPS-c class (*AtCPS/GA1*) or TPS-e class (*AtKS/GA2*) group of TPS genes but instead consist of the other TPS classes (TPS-a and TPS-b). The presence of the gene cluster consisting of TPS-c and TPS-e groups in the rice but not in the Arabidopsis genome could be related to the fact that rice actively produces specific kinds of diterpenes, oryzalexins and momilactones (both of which are derivatives of CDP) as dominant components of phytoalexins, whereas Arabidopsis does not.

The increased copy number of *CPS*-like and *KS*-like genes and their contiguous arrangement suggests that these genes evolved rapidly through gene duplication. In fact, the frequency of nucleotide polymorphism in these regions between the *O. sativa* subsp. *japonica* and *O. sativa* subsp. *indica* genomes is higher than in other regions (data not shown). The frequency of nucleotide polymorphism in these regions could be related to the presence of transposon-like sequences (Fig. 2, B and C). The two gene clusters (*OsCPS2-OsKS5-OsKS6-OsKS7* and *OsCPS4-OsKS4*) contain two and seven copies of transposon-like sequences, respectively. It has been pointed out that retroelements generate instability in genomic regions by promoting recombination and translocation events (Aubourg et al., 2002). Such genomic instability also explains the frequent presence of small pieces of *CPS*-like and *KS*-like sequences in the rice genome (e.g. represented as KS-similar in Fig. 2B).

Based on the contrasting situation between the large copy number of the TPS-a and TPS-b genes and the single copy of GA biosynthetic genes in the Arabidopsis genome, Aubourg et al. (2002) suggested the possibility that the duplication of GA biosynthetic genes might be deleterious for growth and development controlled by GA. However, the rice genome has succeeded in increasing the copy number of *CPS*-like, *KS*-like, and *KO*-like genes. The original of the multiple copies of these genes should be the GA biosynthetic genes because the GA biosynthetic genes are widely shared by monocot and dicot plants, but the genes involved in phytoalexin synthesis are specific in the rice plant. Why did the duplicated genes derived from the GA biosynthetic genes rapidly loose their original GA biosynthesis activities even though the original genes retained them? One possible explanation is that the multiple copies of *CPS*-like, *KS*-like, and *KO*-like genes are deleterious for growth and development, as discussed by Aubourg et al. (2002). Further biochemical studies on these CPS-like, KS-like, and KO-like enzymes are necessary to confirm this proposition.

MATERIALS AND METHODS

Plant Materials

Seeds of wild-type and mutant rice (*Oryza sativa*) were immersed in water for 2 d, grown for 1 month in a greenhouse, and then transplanted to the field.

Sequence Analysis

BLAST search was performed against the rice DNA databases of the Rice Genome Research Program (http://rgp.dna.affrc.go.jp/), the Knowledge-based Oryza Molecular biological Encyclopedia (KOME; http://cdna01.dna.affrc.go.jp/cDNA/), Gramene (http://www.gramene.org/), the Torrey Mesa Research Institute (http://www.tmri.org/index.html), and the Beijing Genomics Institute (http://btn.genomics.org.cn/rice). The deduced amino acid sequences were aligned using the ClustalW program (http://www.ddbj.nig.ac.jp/E-mail/homology.html) with standard parameters, and the phylogenetic tree was obtained with the neighbor-joining method. To map each candidate gene onto the rice genome, linkage analysis was performed using a population of backcrossed inbred lines derived from the cross between Nipponbare (*japonica*) and Kasalath (*indica*) varieties. The linkage was calculated using the MAPMAKER program (Lander et al., 1987). The accession numbers of the sequences used in this paper are indicated in Supplemental Table II.

Mutant Analysis

The effect of GA treatment on the shoot elongation of mutant seedlings was evaluated by microdrop assay (Murakami, 1972). For the mapping of each mutant, linkage analysis was performed using an F_2 population derived from the cross between the mutant (*japonica*) and Kasalath (*indica*) varieties.

GA Analysis

Rice leaves (1 g) were homogenized and extracted with 10 mL of ethyl acetate:methanol (1:1) twice. The extracts were combined, added with 50 ng or 100 ng (for Tanginbozu) of [17,17-$^2H_2$]*ent*-kaurene (purchased from Prof. L. Mander, Australian National University, Canberra, Australia), methylated with excess amount of diazomethane, and concentrated in vacuo. The concentrated extract was then dissolved with 10 mL of ethyl acetate:*n*-hexane (5:95), dried with Na_2SO_4, and half of the solution was applied to Bond Elut SI column (gel size 1 g). The column was flushed with air, and the eluate was concentrated and applied to HPLC analysis with a column of Docosil (250 mm length, 10 mm i.d.; Senshu Scientific, Tokyo). The column was eluted with $CHCl_3$:methanol (5:95) at a flow rate of 2 mL/min, and the eluate was collected as *ent*-kaurene fraction from 45 to 50 min after injection. Gas chromatography-mass spectrometry was performed with an AUTO MASS mass spectrometer (JEOL, Akishima, Japan) connected to a Hewlett-Packard 5890 series II gas chromatograph (Palo Alto, CA) equipped with a column of DB-5 (30 m in length, 0.25 mm i.d., 0.25 μm film thickness; J & W Scientific, Folsom, CA). After the injection, the column oven temperature was maintained at 80°C for 1 min and then increased to 180°C at a rate of 20°C min^{-1}, followed by further increment to 210°C at a rate of 2.5°C min^{-1}. Then it was maintained at 210°C for 10 min and finally increased to 270°C at a rate of 20°C min^{-1}. Authentic *ent*-kaurene was eluted approximately 18.6 min after the injection. The level of *ent*-kaurene was calculated by the area ratio between the peaks at mass-to-charge ratio of 274 (internal standard) and 272 (endogenous). Endogenous GA levels in wild-type and mutant seedlings were analyzed by gas chromatography-selected ion monitoring as described previously (Sakamoto et al., 2001).

Expression Analysis

RT-PCR was performed with DNase-treated total RNAs separately prepared from various organs of rice by using the Advantage RT-for-PCR kit (CLONTECH, Palo Alto, CA). The PCR cycles, in which PCR products were in exponential increase, were determined essentially as described by the supplier. The primer sequences used in this paper are indicated in

Supplemental Table III. These primers specifically amplified the target gene sequences (data not shown). Seedlings of wild-type rice (cv Nipponbare) were UV irradiated for 30 min using a germicidal lamp (15 W) at a distance of 15 cm. After irradiation, these seedlings were incubated at 30°C in the dark for 24 h, and then under light for another 24 h. Total RNAs extracted from elicitor-treated rice cultured cells were kindly provided by Dr. Eiichi Minami (National Institute of Agrobiological Sciences, Tsukuba, Japan). Suspension cultured cells of wild-type rice (cv Nipponbare) were prepared as described previously (Yamada et al., 1993). Purified *N*-acetylchitoheptaose (Kuchitsu et al., 1993) was applied to the medium at a final concentration of 1 mg L^{-1}.

ACKNOWLEDGMENTS

We thank the National Institute of Agrobiological Sciences for providing seed materials. We also thank Dr. Eiichi Minami for providing the RNA samples extracted from elicitor-treated rice cultured cells and Dr. Tomonobu Toyomasu for sharing data before publication.

Received September 22, 2003; returned for revision December 23, 2003; accepted January 9, 2004.

LITERATURE CITED

Aubourg S, Lecharny A, Bohlmann J (2002) Genomic analysis of the terpenoid synthase (*AtTPS*) gene family of *Arabidopsis thaliana*. Mol Genet Genomics **267:** 730–745

Ashikari M, Sasaki A, Ueguchi-Tanaka M, Itoh H, Nishimura A, Swapan D, Ishiyama K, Saito T, Kobayashi M, Khush GS, et al (2002) Loss-of-function of a rice gibberellin biosynthetic gene, *GA20 oxidase* (*GA20ox-2*), led to the rice 'Green Revolution.' Breed Sci **52:** 143–150

Cho E-M, Okada A, Kenmoku H, Otomo K, Toyomasu T, Mitsuhashi W, Sassa T, Yajima A, Yabuta G, Mori K, et al (2004) Molecular cloning and characterization of a cDNA encoding *ent*-cassa-12,15-diene synthase, a putative diterpenoid phytoalexin biosynthetic enzyme, from suspension-cultured rice cells treated with a chitin elicitor. Plant J **37:** 1–8

Evans LT (1993) Crop Evolution, Adaptation and Yield. Cambridge University Press, Cambridge, UK

Feng Q, Zhang Y, Hao P, Wang S, Fu G, Huang Y, Li Y, Zhu J, Liu Y, Hu X, Jia P, Zhang Y, et al (2002) Sequence and analysis of rice chromosome 4. Nature **420:** 316–320

Goff SA, Ricke D, Lan TH, Presting G, Wang R, Dunn M, Glazebrook J, Sessions A, Oeller P, Varma H, Hadley D, Hutchison D, et al (2002) A draft sequence of the rice genome (*Oryza sativa* L. ssp. *japonica*). Science **296:** 92–100

Hedden P, Kamiya Y (1997) Gibberellin biosynthesis: enzymes, genes and their regulation. Annu Rev Plant Physiol Plant Mol Biol **48:** 431–460

Hedden P, Phillips AL (2000) Gibberellin metabolism: new insights revealed by the genes. Trends Plant Sci **5:** 523–530

Hedden P, Phillips AL, Rojas MC, Carrera E, Tudzynski B (2002) Gibberellin biosynthesis in plants and fungi: A case of convergent evolution? J Plant Growth Regul **20:** 319–331

Ikeda A, Ueguchi-Tanaka M, Sonoda Y, Kitano H, Koshioka M, Futsuhara Y, Matsuoka M, Yamaguchi J (2001) *slender rice*, a constitutive gibberellin response mutant, is caused by a null mutation of the *SLR1* gene, an ortholog of the height-regulating gene *GAI/RGA/RHT/D8*. Plant Cell **13:** 999–1010

Itoh H, Tatsumi T, Sakamoto T, Otomo K, Toyomasu T, Kitano H, Ashikari M, Ichihara S, Matsuoka M (2004) A rice semi-dwarf gene, *Tan-Ginbozu* (*D35*), encodes the gibberellin in biosynthesis enzyme, *ent*-kaurene oxidase. Plant Mol Biol **55:** in press

Itoh H, Ueguchi-Tanaka M, Sentoku N, Kitano H, Matsuoka M, Kobayashi M (2001) Cloning and functional analysis of gibberellin 3β-hydroxylase genes that are differently expressed during the growth of rice. Proc Natl Acad Sci USA **98:** 8909–8914

Kato H, Kodama O, Akatsuka T (1995) Characterization of an inducible P450 hydroxylase involved in the rice diterpene phytoalexin biosynthetic pathway. Arch Biochem Biophys **316:** 707–712

Kikuchi S, Satoh K, Nagata T, Kawagashira N, Doi K, Kishimoto N, Yazaki J, Ishikawa M, Yamada H, Ooka H, et al (2003) Collection, mapping, and annotation of over 28,000 cDNA clones from *japonica* rice. Science **301:** 376–379

Kobayashi M, Sakurai A, Saka H, Takahashi N (1989) Quantitative analysis of endogenous gibberellins in normal and dwarf cultivars of rice. Plant Cell Physiol **30:** 963–969

Kuchitsu K, Kikuyama M, Shibuya N (1993) *N*-acetylchitooligosaccharides, biotic elicitor for phytoalexin production, induce transient membrane depolarization in suspension-cultured rice cells. Protoplasma **174:** 79–81

Lander ES, Green P, Abrahamson J, Barlow A, Daly MJ, Lincoln SE, Newburg L (1987) MAPMAKER: an interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. Genomics **1:** 174–181

Lester DR, Ross JJ, Smith JJ, Elliott RC, Reid JB (1999) Gibberellin 2-oxidation and the *SLN* gene of *Pisum sativum*. Plant J **19:** 65–73

Martin DN, Proebsting WM, Hedden P (1999) The *SLENDER* gene of pea encodes a gibberellin 2-oxidase. Plant Physiol **121:** 775–781

Mohan RS, Yee NK, Coates RM, Ren YY, Stamenkovic P, Mendez I, West CA (1996) Biosynthesis of cyclic diterpene hydrocarbons in rice cell suspensions: conversion of 9,10-*syn*-labda-8(17),13-dienyl diphosphate to 9β-pimara-7,15-diene and stemar-13-ene. Arch Biochem Biophys **330:** 33–47

Murakami Y (1972) Dwarfing genes in rice and their relation to gibberellin biosynthesis. *In* DJ Carr, ed, Plant Growth Substances. Springer-Verlag, Berlin, pp 166–174

Ogawa S, Toyomasu T, Yamane H, Murofushi N, Ikeda R, Morimoto Y, Nishimura Y, Omori T (1996) A step in the biosynthesis of gibberellins that is controlled by the mutation in the semi-dwarf rice cultivar Tan-ginbozu. Plant Cell Physiol **37:** 363–368

Sakai M, Sakamoto T, Saito T, Matsuoka M, Tanaka H, Kobayashi M (2003) Expression of novel rice gibberellin 2-oxidase gene is under homeostatic regulation by biologically active gibberellins. J Plant Res **116:** 161–164

Sakamoto T, Kobayashi M, Itoh H, Tagiri A, Kayano T, Tanaka H, Iwahori S, Matsuoka M (2001) Expression of a gibberellin 2-oxidase gene around the shoot apex is related to phase transition in rice. Plant Physiol **125:** 1508–1516

Sasaki A, Ashikari M, Ueguchi-Tanaka M, Itoh H, Nishimura A, Swapan D, Ishiyama K, Saito T, Kobayashi M, Khush GS, et al (2002a) A mutant gibberellin-synthesis gene in rice. Nature **416:** 701–702

Sasaki T, Matsumoto T, Yamamoto K, Sakata K, Baba T, Katayose Y, Wu J, Niimura Y, Cheng Z, Nagamura Y, et al (2002b) The genome sequence and structure of rice chromosome 1. Nature **420:** 312–316

Schomburg FM, Bizzell CM, Lee DJ, Zeevaart JAD, Amasino RM (2003) Overexpression of a novel class of gibberellin 2-oxidases decreases gibberellin levels and creates dwarf plants. Plant Cell **15:** 151–163

Toyomasu T, Kawaide H, Sekimoto H, Numers C, Phillips AL, Hedden P, Kamiya Y (1997) Cloning and characterization of a cDNA encoding gibberellin 20-oxidase from rice (*Oryza sativa*) seedlings. Physiol Plant **99:** 111–118

Wickham KA, West CA (1992) Biosynthesis of rice phytoalexins: identification of putative diterpene hydrocarbon precursors. Arch Biochem Biophys **293:** 320–332

Yamada A, Shibuya N, Kodama O, Akatsuka T (1993) Induction of phytoalexin formation in suspension-cultured rice cells by *N*-acetylchitooligosaccharides. Biosci Biotechnol Biochem **57:** 405–409

Yu J, Hu S, Wang J, Wong GK, Li S, Liu B, Deng Y, Dai L, Zhou Y, Zhang X, et al (2002) A draft sequence of the rice genome (*Oryza sativa* L. ssp. *indica*). Science **296:** 79–92

CORRECTION

Vol. 134: 1642–1653, 2004

Sakamoto T., Miyura K., Itoh H., Tatsumi T., Ueguchi-Tanaka M., Ishiyama K., Kobayashi M., Agrawal G.K., Takeda S., Abe K., Miyao A., Hirochika H., Kitano H., Ahikari M., and Matsuoka M. An Overview of Gibberellin Metabolism Enzyme Genes and Their Related Mutants in Rice.

The authors regret two typographical errors in Table I:

The Entry Name for *OsCPS1* should be AP004872 (not Ap004572).

The Chromosome Location for *OsCPS1* should be Chr. 2, 49 cM (not Chr. 2, 2.49 cM).