Gibberellin 20-Oxidase Gene *OsGA20ox3* Regulates Plant Stature and Disease Development in Rice

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Gibberellin (GA) 20-oxidase (GA20ox) catalyses consecutive steps of oxidation in the late part of the GA biosynthetic pathway. A T-DNA insertion mutant (17S-14) in rice, with an elongated phenotype, was isolated. Analysis of the flanking sequences of the T-DNA insertion site revealed that an incomplete T-DNA integration resulted in enhanced constitutively expression of downstream *OsGA20ox3* in the mutant. The accumulation of bioactive GA₁ and GA₄ were increased in the mutant in comparison with the wild-type plant. Transgenic plants overexpressing *OsGA20ox3* showed phenotypes similar to those of the 17S-14 mutant, and the RNA interference (RNAi) lines that had decreased *OsGA20ox3* expression exhibited a semidwarf phenotype. Expression of *OsGA20ox3* was detected in the leaves and roots of young seedlings, immature panicles, anthers, and pollens, based on β -glucuronidase (GUS) activity staining in transgenic plants expressing the *OsGA20ox3* promoter fused to the *GUS* gene. The *OsGA20ox3* RNAi lines showed enhanced resistance against rice pathogens *Magnaporthe oryzae* (causing rice blast) and *Xanthomonas oryzae* pv. *oryzae* (causing bacterial blight) and increased expression of defense-related genes. Conversely, *OsGA20ox3*-overexpressing plants were more susceptible to these pathogens comparing with the wild-type plants. The susceptibility of wild-type plants to *X. oryzae* pv. *oryzae* was increased by exogenous application of GA₃ and decreased by S-3307 treatment. Together, the results provide direct evidence for a critical role of *OsGA20ox3* in regulating not only plant stature but also disease resistance in rice.

Gibberellins (GA) are a group of tetracyclic diterpenoid compounds, some of which play important roles in regulating many aspects of plant growth and development, such as seed germination, stem elongation, leaf expansion, and flower and fruit development (MacMillan 2002; Silverstone and Sun 2000). Genetic and biochemical studies have revealed many genes that participate in GA biosynthesis or signal transduction pathways (Hedden and Phillips 2000; Hedden et al. 2002; Kaneko et al. 2003; Sakamoto et al. 2004). GA are

synthesized from *trans*-geranylgeranyl diphosphate (GGDP), which is a universal precursor for diterpenoids. *ent*-Kaurene is produced by sequential cyclizations of GGDP via two kinds of diterpene cyclases in proplastid, *ent*-copalyl diphosphate synthase (CPS) and *ent*-kaurene synthase (KS). *ent*-Kaurene is then converted to GA₁₂ via two steps of oxidations by cytochrome P450 monooxygenases, *ent*-kaurene oxidase (KO) and *ent*-kaurenoic acid oxidase (KAO), in the endoplasmic reticulum membrane. Bioactive GA₁ and GA₄ are synthesized from GA₁₂ through two parallel pathways (i.e., early-13-hydroxylation and non-13-hydroxylation pathways) by two kinds of 2-oxoglutarate-dependent dioxygenases in the cytoplasm, GA 20-oxidases (GA20ox) and GA 3-oxidases (GA3ox). GA20ox catalyzes the sequential oxidation of C-20 from a methyl group to its removal as CO₂, producing C₁₉-GA (GA₉ and GA₂₀). Subsequently, the introduction of a β -hydroxyl group by GA3ox converts the inactive C₁₉-GA precursors to bioactive GA (Hedden and Phillips 2000; Olszewski et al. 2002).

The enzymes catalyzing early steps in GA biosynthetic pathway, such as CPS, KS, and KO, are generally encoded by one or two genes. For example, there are four *CPS* genes identified in the rice genome, including a pseudogene, *OsCPS3*. However, only *OsCPS1* is shown to be involved in GA biosynthesis (Sakamoto et al. 2004). *OsCPS2* and *OsCPS4* participate in the biosynthesis of phytoalexins (Otomo et al. 2004; Priscic et al. 2004; Sakamoto et al. 2004). A loss-of-function mutation in *OsCPS1* causes serious dwarfism. The enzymes catalyzing later steps in the GA biosynthetic pathway, such as GA20ox and GA3ox, are encoded by small gene families. The *semi-dwarf1* (*sd1*) mutant, known to be mutated in the “Green Revolution Gene”, shows a semidwarfism phenotype with strong lodging resistance and increased yields compared with the wild-type (WT) progenitor (Wolfgang et al. 2002). The *SD1* encodes an *OsGA20ox2* gene located on chromosome 1 in rice. RNA interference (RNAi)-mediated suppression of *OsGA20ox2* transcript also causes a semidwarf phenotype in rice, similar to the phenotype of *sd1* (Qiao et al. 2007). There are four *OsGA20ox*-like genes in the rice genome and they differ in their expression patterns (Sakamoto et al. 2004), suggesting that they may play different roles. The *OsGA20ox1* gene has been characterized based on its overexpression, which causes a tall and GA-overproduction phenotype (Oikawa et al. 2004). RNAi-mediated suppression of *OsGA20ox1* expression results in phenotypes that are similar to those of *sd1*, indicating that *OsGA20ox1* and *OsGA20ox2* probably have complementary function in GA synthesis and regulation of plant stature (Oikawa et al. 2004). In *Arabidopsis*, *AtGA20ox1* and *AtGA20ox2*, two of the five known *GA20ox* genes, act partially redundantly to promote growth and development (Rieu et al. 2008).

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Plant height is one of the most important traits in crop breeding because of its relevance to lodging resistance. A number of dwarf or tall mutants of rice have been isolated, and some of them have been characterized as enzymes involved in GA biosynthesis or components in GA signaling pathways. The GA receptor mutant *gibberellin-insensitive dwarf1* (*gid1*) shows serious dwarfism and the mutant phenotype cannot be restored by application of exogenous GA. The WT *GID1* receptor protein directly binds to active GA and, subsequently, interacts with DELLA transcriptional repressors. The GA-*GID1*-bound DELLA proteins are recognized and ubiquitinated by an E3 ubiquitin ligase (Skp1, Cullin, and F-box [SCF]^{*GID2/SLY1*}), leading to degradation of DELLA proteins through the proteasome pathway (Ueguchi-Tanaka et al. 2005, 2007). Degradation of DELLA repressors activates GA action. *Arabidopsis* contains multiple DELLA proteins, whereas rice has only one, called SLENDER RICE1 (*SLR1*). The *slr1* mutant exhibits constitutive GA signaling and responses (Ikeda et al. 2001).

Although GA are widely recognized as phytohormones that play important roles in plant growth and development, emerging evidence implicates GA in the processes of pathogen infection. In fact, GA was originally identified from the necrotrophic fungus *Gibberella fujikuroi* (*Fusarium moniliforme*), which causes the *bakanae* or foolish-seedling disease of rice (Yabuta and Sumiki 1938). Among many symptoms, the infected plants are slender, caused by excessive growth of the stem, and often are incapable of supporting their own weight, topple over, and die (hence "foolish seedling disease"). The mutant *elongated uppermost internode* (*eui*), with increased accumulation of active GA (Zhu et al. 2006), has compromised disease resistance to *Xanthomonas oryzae* pv. *oryzae* and *Magnaporthe oryzae* (Yang et al. 2008). The *gid1* mutant, which exhibited typical GA-deficient phenotypes despite elevated GA contents, shows increased tolerance to cold stress as well as resistance to rice blast fungus *M. oryzae* (Tanaka et al. 2006). Interestingly, the *OsCPS1* transcript is downregulated, whereas expression of *OsCPS2* and *OsCPS4* in the phytoalexin biosynthesis pathway is upregulated in *gid1* (Tanaka et al. 2006). These results suggest that GA homeostasis participates in responses to pathogen attack.

In this report, we isolated a T-DNA insertion mutant (17S-14) in rice which showed a tall and elongated phenotype. Further analysis revealed an irregular T-DNA integration that resulted in a *Cauliflower mosaic virus* (CaMV) 35S promoter located upstream of the *OsGA20ox3* gene in the mutant. Transgenic overexpression and knockdown of *OsGA20ox3* expression showed elongated and semidwarf phenotypes, respectively, and exhibited altered resistance to pathogen infections. Our results provide direct evidence for a critical dual role of *OsGA20ox3* in regulating plant stature and disease resistance in rice.

RESULTS

Characterization of rice T-DNA insertion mutant 17S-14 with GA overproduction phenotypes.

In a project to examine the role of the wall-associated kinase 2 (*OsWAK2*) gene in disease resistance, the full-length coding sequence of *OsWAK2* was isolated and inserted into a modified pCambia1301 vector under the control of a CaMV35S promoter (Li et al. 2009). The *OsWAK2* overexpression construct was used to transform rice 'Aichi Asahi'. One transformant, 17S-14, differed in appearance from all other transgenic lines harboring the *CaMV35S:OsWAK2* construct. Genetic analysis showed that the T₁ plants yielded a segregation of 17 tall plants with resistance to hygromycin (Hyg) and 6 Hyg-sensitive plants of normal height, indicating dominant inheri-

tance of the elongated phenotype. Line 17S-14 was approximately 55 ± 4 cm taller than WT parents (104 ± 3 cm) under the natural long-day conditions in Beijing, China. The mature plants of line 17S-14 have a taller stature, longer spikes, longer internodes, and more elongated internodes than WT plants (Fig. 1B and C). Seed of line 17S-14 germinated earlier and seedlings grew faster than its WT parents (Fig. 1A and B). These characteristics were similar to those of rice plants treated with active GA or a GA-overproduction mutant such as B142 (Oikawa et al. 2004), suggesting that 17S-14 is a GA-overproduction or hyper-responsive mutant.

We investigated the responses of the 17S-14 mutant to GA₃, an active GA, and S-3307, a GA biosynthesis inhibitor (similar to uniconazole). Germinated seed of the WT and the 17S-14 mutant were transferred to one-half Murashige-Skoog medium containing various levels of GA₃ or S-3307 for growth. The growth of 17S-14 seedlings was enhanced by treatment with GA₃, similar to the response of the WT plants applied with GA₃ (Fig. 2A and C). In addition, inhibition of growth was observed in both of the 17S-14 mutant and WT plants treated with 5 μM S-3307 (Fig. 2A and B). Normal responses of the 17S-14 mutant to GA and GA biosynthesis inhibitor suggest that the GA signal-transduction pathway is maintained intact.

Isolation of the flanking sequences of the T-DNA insertion site from the 17S-14 mutant.

Southern blot analysis revealed a single T-DNA insertion in the 17S-14 line using the Hyg phosphotransferase gene as a hybridization probe (Fig. 3A), suggesting that the mutant phenotype may be caused by the T-DNA insertion. However, we were unsuccessful in using the thermal asymmetrical interlaced polymerase chain reaction (PCR) method to isolate the flanking sequences at the T-DNA insertion site in the 17S-14 mutant (Liu et al. 1995). Next, we constructed a genomic library of the 17S-14 mutant and screened it using the Hyg gene as a probe. The flanking sequences were obtained by sequencing six positive clones and found to correspond to the sequence of the BAC clone OsJNBa0050F10 (accession number AP005840) from chromosome 7. The site of T-DNA insertion is located 385 bp upstream of a possible translation start site of the *OsGA20ox3* gene (Fig. 3B). Sequence analysis revealed that the T-DNA insert in the 17S-14 genome was incomplete, containing a deletion from the middle of the *OsWAK2* transgene to the left border of TDNA (Fig. 3C). The deletion resulted in loss of not only the 3' half of the *OsWAK2* gene but also the β-glucuronidase (*GUS*) reporter gene in the T-DNA vector, agreeing with the result of undetectable band using the *GUS* gene as probe in the Southern blot analysis (Fig. 3A).

Increased expression of the *OsGA20ox3* gene and accumulation of active GA in the 17S-14 mutant.

The *GA20ox* is a key enzyme of the later steps of the GA biosynthesis pathway. Four *GA20ox*-like genes exist in the rice genome (Sakamoto et al. 2004). The deduced amino acid sequence of *OsGA20ox3* contains the conserved amino acid sequences of NHYPPCRQP for binding the 2-oxoglutarate substrate, three histidine (His) residues for binding Fe²⁺, and the amino acid sequence LPWKET for binding GA substrates (Sakamoto et al. 2004). Because two CaMV35S promoters were located very close to *OsGA20ox3* gene, we speculated that the transcription of *OsGA20ox3* might be enhanced in mutant 17S-14 plants, resulting in increased GA biosynthesis. Indeed, by reverse-transcriptase (RT)-PCR analysis, we detected an elevated level of *OsGA20ox3* transcript in the 17S-14 mutant compared with that of WT plants (Fig. 3D). Next, we conducted quantitative analysis of endogenous GA by using a liquid chromatography mass spectrometry (LC-MS) method.

Fig. 1. Characteristics of the 17S-14 plant. **A**, Seed germination of wild-type (WT) and the 17S-14 mutant (MT). **B**, Statures of the WT and MT at 2-week-old (left), flowering (middle), and mature (right) stages. **C**, Lengths of spikes and internodes (In-1 to In-9) of WT and MT were measured from six plants. Error bars indicate standard errors of the mean. Bars = 1 cm (A) and 10 cm (B). Asterisk indicates significant differences between the control and the overexpressing lines (* indicates $P < 0.05$, Fieldman Rank Sums test).

The active GA molecules of GA₁ and GA₄ accumulated higher in 17S-14 plants compared with the WT plants (Table 1). Taken together, these results indicated that increased GA accumulation is likely a cause of the excessive shoot elongation in the 17S-14 mutant plant.

Developmental regulation of the *OsGA20ox3* gene.

To examine the expression of *OsGA20ox3* in different organs, RT-PCR analysis of the *OsGA20ox3* transcript was performed using total RNA isolated from various organs of the WT and 17S-14 plants. *OsGA20ox3* was broadly expressed in vegeta-

tive organs of 2-week-old seedlings, including leaves, sheaths, and roots (Fig. 4A). The *OsGA20ox3* transcript was most abundant in the panicles and not detected in leaves, sheaths, culms, or roots at the heading stage of rice. In contrast, the *OsGA20ox3* transcript accumulated at high levels in all organs examined in the 17S-14 plants (Fig. 4A).

To further investigate the expressing patterns of *OsGA20ox3* in detail, we fused the *OsGA20ox3* promoter with the *GUS* gene (*P_{OsGA20ox3}::GUS*) and transformed it into Aichi Asahi rice plants. A representative *GUS* activity pattern is shown in Figure 4B. *GUS* activity was detected in transgenic callus, germi-

Fig. 2. Effect of GA₃ or S-3307 treatment on seedling growth. **A**, Seedlings of wild-type (WT) and the 17S-14 mutant (MT) were cultured on one-half Murashige-Skoog solid medium (1% agar) containing different concentrations of S-3307 or GA₃ for 10 days. The second leaf sheath lengths were measured and the mean values from six plants were shown for **B**, S-3307 and **C**, GA₃ treatments. Error bars indicate standard errors of the mean. Bar = 2 cm.

nating seed, and sheaths and roots of seedlings (Fig. 4Ba to c). Furthermore, GUS activity was found in young panicles, anthers, and pollens (Fig. 4Bd to j) but not in leaves, sheaths, roots, or culms at the heading stage (data not shown). Therefore, the results obtained by GUS staining were in agreement with the expressing patterns revealed by RT-PCR analysis.

The *OsGA20ox3* gene plays a positive role in plant stature.

To investigate the biological functions of *OsGA20ox3*, the coding region of *OsGA20ox3* driven by the CaMV35S promoter (CaMV35S:*OsGA20ox3*) was introduced into rice (Aichi Asahi). In addition, an RNAi construct was transformed into rice. Of 21 transformants harboring the *CaMV35S:OsGA20ox3*

Fig. 3. Characterization of the T-DNA insertion in line 17S-14. **A**, Southern blot analysis using the genomic DNA (20 µg per each lane) from wild-type (WT) and the 17S-14 mutant (MT) plants. From lane 3 to 5, the DNA from MT was digested with *Hind*III, *Eco*RI, and *Apa*I, respectively. The DNA from WT (lane 2) and the pCambia1301 vector (lane 6) digested with *Hind*III were used as the negative and positive controls, respectively. *Hind*III-fragmented λ DNA was used as the size markers (lane 1). Fragments of hygromycin (*Hyg*) (left) or β-glucuronidase (*GUS*) (right) were labeled with ³²P and used as hybridization probes. **B**, Diagram of the T-DNA insertion site in the 17S-14 plant. The T-DNA was located in the BAC clone OSJNBa0050F10 (contains 151,986 bp) at the position of 78,409 bp and located at 385 bp upstream of the potential translation start site of *OsGA20ox3*. **C**, Diagram shown the part of the original *OsWAK2* transgene construct missing in the 17S-14 plant. **D**, Expression of *OsGA20ox3* was analyzed by the reverse-transcriptase polymerase chain reaction (PCR) method using the total RNA isolated from 2-week-old seedlings. The PCR products were separated by electrophoresis in a 1.2% (wt/vol) agarose gel and the DNA bands were visualized using an Alphaimager 2200 system. The experiments were repeated 10 times and similar results were obtained. The *OsActin* gene was used as an internal standard.

construct, 18 showed tall, elongated internode phenotypes, similar to that of 17S-14 mutant plants (Fig. 5A). Of 15 RNAi lines, 3 showed semidwarf phenotypes (Fig. 5A). Transcription analyses revealed that *OsGA20ox3* expression was enhanced in the overexpressing lines OV4 and OV5, which showed elongated seedling phenotypes. In contrast, *OsGA20ox3* expression was significantly suppressed in the RNAi lines RI9 and RI11, which show a semidwarf phenotype, compared with the WT and the RNAi lines RI8 and RI10 plants, which exhibited a relatively

Table 1. Endogenous concentration of gibberellin (GA)₁ and GA₄ in wild-type and 17S-14 mutant seedlings^a

	Concentration (ng g ⁻¹ fresh weight)	
	GA ₁	GA ₄
Wild-type	0.07 ± 0.02	1.73 ± 0.08
17S-14 mutant	6.70 ± 1.31	20.49 ± 0.51

^a GA were extracted from entire seedlings of the 17S-14 mutant and its progenitor with two biological replicates and analyzed by liquid chromatography mass spectrometry.

normal growth phenotype (Fig. 5B and C). In addition, we examined the effects of *OsGA20ox3* RNAi and overexpression on the expression of its close homologs. We found that the expression of *OsGA20ox3* homologs was not markedly different in the *OsGA20ox3*-overexpressing plants compared with that in the WT plants, except that *OsGA20ox2* was modestly elevated (approximately twofold) in line 17S-14 and, to a lesser extent, also in some of the *OsGA20ox2* overexpression lines. In contrast, alteration of gene expression was highly specific for *OsGA20ox3* in the RNAi lines of semidwarves, without significant effect on the level of homologous *OsGA20ox1*, *OsGA20ox2*, and *OsGA20ox4* (Fig. 5D). The results provide the first evidence that *OsGA20ox3* plays a positive role in plant stature of rice and suggest that *OsGA20ox3* overexpression is likely the reason for the elongated phenotype of 17S-14 mutant plants.

Increase *OsGA20ox3* expression enhances rice susceptibility to pathogens.

Accumulating data showed that GA is also involved in disease responses in rice (Tanaka et al. 2006; Yang et al. 2008).

Fig. 4. Developmental expression patterns of *OsGA20ox3*. **A**, Expression patterns of *OsGA20ox3* in wild-type (WT) and 17S-14 mutant (MT) plants were examined by reverse-transcriptase polymerase chain reaction. Total RNAs were extracted from various tissues of 2-week-old seedlings (lanes 1 to 4: leaves, upper part of sheaths, lower part of sheaths, and roots, respectively) and at the heading stage (lanes 5 to 10: panicles, leaves, sheaths, upper part of culms, lower part of culms, and roots, respectively). *OsActin* gene was used as an internal standard. **B**, **a**, Histochemical β -glucuronidase (GUS) staining of *P_{OsGA20ox3}::GUS* transgenic calli. GUS activity in **b**, 3-day-old and **c**, 10-day-old seedlings; **d**, young and **f**, flowering panicles; **h**, anthers; and **j**, pollens. **e**, **g**, and **i**, GUS staining of WT plants used as the corresponding controls. Bars = 1 cm (a to f), 0.1 cm (g and h), and 20 μ m (i and j).

Therefore, we investigated whether *OsGA20ox3* plays a role in responses to pathogen infection of rice. Transgenic plant overexpressing or underexpressing *OsGA20ox3*, 17S-14 mutant, and their corresponding WT plants were inoculated with rice

blast fungal strain MS220. The 17S-14 mutant exhibited more severe disease symptoms, including larger lesions, than the WT plants (Fig. 6A and B). Similarly, *OsGA20ox3*-overexpressing lines OV4 and OV5 were more susceptible to the rice blast

Fig. 5. Characterization of *OsGA20ox3* overexpression and RNA interference (RNAi) plants. **A**, Comparison of the statures of *OsGA20ox3* overexpression (OV4) and RNAi (RI11 and RI16) lines (T_1) with wild-type (WT) and 17-14S mutant (MT) plants. **B**, Plant heights and standard errors of six plants are shown. Analysis of gene expression was performed by **C**, reverse-transcriptase polymerase chain reaction (RT-PCR) and **D**, quantitative PCR (qPCR) using 2-week-old seedlings of WT, MT, and overexpressing (OV4 and OV5) and RNAi (RI8–RI11) lines at the T_2 generation. Actin (*OsActin*) and ubiquitin (*UBQ*) genes were used as internal standards for RT-PCR and qPCR, respectively. Mean values from three repeats are shown. Error bars indicate standard errors of the mean. Bar = 10 cm.

Fig. 6. OsGA20ox3 plays a negative role in rice resistance to *Magnaporthe oryzae* and *Xanthomonas oryzae* pv. *oryzae*. Three-week-old rice plants were inoculated with conidial suspensions (2×10^5) of *M. oryzae* MS220 by spray. **A**, Photos were taken at 7 days postinoculation (dpi). **B**, Disease index was evaluated by counting the number of lesions on the third leaves of the rice plants. Mean values and standard errors were from 8 plants. **C**, Plants (approximately 6 weeks old) were inoculated with *X. oryzae* pv. *oryzae* PXO99 by the leaf-clipping method and photos were taken at 12 dpi. **D**, Two-week-old seedlings were sprayed with 60 μ M GA₃, 20 μ M GA synthetic inhibitor S-3307, or 0.1% ethanol as the control six times at an interval of 4 days each. Two days after the last spray, the plants were inoculated with *X. oryzae* pv. *oryzae* as described in C. **E** and **F**, Lesion length was measured and shown as mean values and standard errors from 10 plants. Bar = 5 cm.

pathogen than the WT. Conversely, the *OsGA20ox3* RNAi lines RI9 and RI11 developed less disease than the WT plants (Fig. 6A and B).

We also inoculated these plants with the bacterial pathogen *X. oryzae* pv. *oryzae* race 6 (strain PXO99) using the leaf-clipping method. The disease lesions were longer in the 17S-14 mutant plants than those in the WT plants (Fig. 6C and E). In contrast, suppression of *OsGA20ox3* expression in RNAi plants was correlated with less lesion development, whereas the overexpressing lines showed more severe disease than the WT plants. To further investigate the effect of GA on disease responses, the 17S-14 and WT plants were treated with GA₃ or GA synthesis inhibitor S-3307. The lesion development was inhibited in both the WT and 17S-14 plants by S-3307 (Fig. 6D and F). In contrast, the lesion symptom was enhanced by GA₃ in both the WT and 17S-14 plants.

Next, we examined the expressions of genes related to defense reactions. We found that expression of pathogenesis-related (*PR*) genes (*OsPR1b* and *OsPR10* [*PBZI*]) was induced by inoculation of *M. oryzae* in all plants but the induction was lower in 17S-14 and *OsGA20ox3*-overexpression line OV4 and higher in the RNAi line RI9 compared with the WT plants (Fig. 7A and B). *OsGA20ox3* and its close homologous *OsGA20ox2* transcript level was not changed significantly in the WT or the RNAi line RI9 in response to infection by the rice blast fungus, and only slight variations of *OsGA20ox3* expression were detected in 17S-14 and overexpressing plants in response to the pathogen treatment (Fig. 7C). Increase of *OsWRKY71* expression has been reported to enhance disease resistance in rice (Liu et al. 2006). We found that both the basal and induced levels of

the *OsWRKY71* transcript were suppressed in 17S-14 and *OsGA20ox3*-overexpressing plants and increased slightly in the RNAi line RI9 in comparison with the WT (Fig. 7D). Finally, diterpene cyclases, *ent*-copalyl diphosphate (*ent*-CDP) synthase (*OsCPS2*/*OsCyc2*), and *syn*-CDP synthase (*OsCPS4*/*OsCyc1*) catalyze the production of precursors leading to synthesis of phytoalexins (Kanno et al. 2006). We found that the *OsCPS2* and *OsCPS4* transcript levels were higher in the *OsGA20ox3* RNAi line RI9 than those of the WT, 17S-14, and *OsGA20ox3*-overexpressing plants (Fig. 7E and F). Altogether, these results show that alterations in *OsGA20ox3* expression affect the expression of defense response genes that have been shown to be associated with rice resistance to pathogens.

DISCUSSION

In this study, we aimed at transgenically expressing the *OsWAK2* gene to assess the role of *OsWAK2* in disease resistance, and we isolated a rice mutant, 17S-14, that showed a GA-constitutive response phenotype. However, by accident, the isolation of this mutant, caused by a partial T-DNA insertion carrying a truncated *OsWAK2*, led us to discover that previously unknown role of *OsGA20ox3* in GA biosynthesis. In addition, we found that the 17S-14 mutant was more susceptible to two major rice pathogens, the rice blast fungus *M. oryzae* and the rice blight bacterium *X. oryzae* pv. *oryzae*. Collectively, our results add to a growing body of evidence that GA plays a dual role in promoting growth and disease susceptibility in plants.

Gain-of-function mutations can be misleading but sometimes can also be an effective method for indicating gene func-

Fig. 7. *OsGA20ox3* negatively regulates expression of defense-related genes. Three-week-old rice plants were inoculated with *Magnaporthe oryzae* and sampled at the time points indicated. Samples collected at 0 and 24 h post-pathogen inoculation were used for quantitative polymerase chain reaction analysis of the expression of **A**, *OsPR1b*; **B**, *OsPR10*; **C**, *OsGA20ox3*; **D**, *OsWRKY71*; **E**, *OsCPS2*; and **F**, *CPS4*. The ubiquitin (*UBQ*) gene was used as internal standard. Mean values and standard errors from three repeats are shown.

tion, especially for genes that are members of a multi-gene family with redundant functions (Nakamura et al. 2007). Our analysis of the 17S-14 mutant illustrates the utility of a gain-of-function mutation in the elucidation of the *OsGA20ox3* function in GA biosynthesis. The enhanced expression of the *OsGA20ox3* transcript, due to insertion of CaMV35S promoter-carrying T-DNA upstream of this gene, was the first indication that the constitutive GA response phenotype in the 17S-14 mutant might be caused by overexpression of *OsGA20ox3*, which belongs to a four-member gene family. We were able to confirm the causal effect of *OsGA20ox3* overexpression in the 17S-14 mutant by independently producing and characterizing *OsGA20ox3*-overexpressing and RNAi-silenced transgenic rice plants. Our studies demonstrate that *OsGA20ox3* plays a role similar to that of its homologous *OsGA20ox1* and *SD1* (*OsGA20ox2*) gene in GA biosynthesis and regulating plant growth.

In many cases, the sites where bioactive GA are produced associate with the sites of their action (Kaneko et al. 2003; Yamaguchi 2008). In this study, expression of *OsGA20ox3* was observed in leaves, sheaths, and roots of 2-week-old seedlings but not in these tissues at the heading stage of rice (Fig. 4A), suggesting that *OsGA20ox3* participates in the process of active growth in young plants. GUS activity staining also showed *OsGA20ox3* specifically expressed in growing tissues and in young panicles, anthers, and pollens (Fig. 4B). Bioactive GA are accumulated at a high level in the anthers of many plants (Hasegawa et al. 1995) and considered to be a source for other floral organs (Kaneko et al. 2003; Weiss et al. 1995). Previous studies have shown that *OsGA20ox1* and *OsGA3ox1* are expressed specifically in the tapetum of the anther and the epithelium of the embryo, suggesting the role of these genes in preferential production of GA₄ that functions as a paracrine for other tissues and organs, whereas *OsGA20ox2* and *OsGA3ox2* are considered to be another set of enzymes that preferentially produce GA₁ during the vegetative and reproductive stages (Kaneko et al. 2003). The expression profile of *OsGA20ox3* suggests that *OsGA20ox3* can complement the function of *OsGA20ox1* and *OsGA20ox2* in GA biosynthesis, supported by increased accumulation of both GA₁ and GA₄ in the 17S-14 mutant (Table 1). GA play important roles in regulating flowering and development of floral organs (Aya et al. 2009; Chhun et al. 2007). The GA-deficient mutant of rice causes impaired pollen germination and pollen growth. We also noticed that the *OsGA20ox3* RNAi lines of semidwarf plants set much fewer seeds than the WT plants (data not shown). The results demonstrated that gene *OsGA20ox3* plays positive roles in growth and reproduction, similar to *OsGA20ox1* and *OsGA20ox2*.

Emerging evidence suggests that GA are involved not only in plant development but also in disease resistance. In fact, GA were first isolated from the fungal pathogen *G. fujikuroi* (Yabuta and Sumiki 1938), which causes super-elongated rice. The rice *eui* mutant, which hyperaccumulates active GA, displays compromised disease resistance to *X. oryzae* pv. *oryzae* and *M. oryzae* (Yang et al. 2008), suggesting that GA acts in pathogenicity. Conversely, the GA perception-deficient mutant *gid1* shows enhanced resistance to *M. oryzae* (Tanaka et al. 2006). In *Arabidopsis*, the mutant with loss-of-function in DELLA proteins which exhibits constitutive activation of GA signaling, is more resistant to biotrophic bacterial pathogens but hyper-susceptible to necrotrophic pathogens (Navarro et al. 2008; Robert-Seilantantz et al. 2007). In this study, we found that the 17S-14 mutant is more susceptible to bacterial blight and rice blast than the WT plants (Fig. 6A and C). Furthermore, suppression of GA synthesis by a GA biosynthetic inhibitor increased resistance to *X. oryzae* pv. *oryzae*. In contrast, exoge-

nous GA₃ treatment made plants more susceptible to *X. oryzae* pv. *oryzae* (Fig. 6C). These results not only are consistent with the notion that GA potentially can be effective virulence factors for certain plant pathogens, such as *G. fujikuroi*, but also suggest that a high level accumulation of GA during certain stages of normal plant development or under certain environmental conditions that promote rapid plant growth may be accompanied by heightened disease susceptibility. As such, GA-mediated growth-defense conflict likely has significant practical implications in agriculture.

A number of observations indicate that crosstalks between phytohormones are important for coordinating growth and development with adaptation to environmental stresses. Auxin promotes the accumulation of bioactive GA (Frigerio et al. 2006), whereas GA is required for proper auxin transport (Willige et al. 2011). Suppression of auxin signaling is thought to be part of the salicylic acid (SA)-mediated plant defense mechanism in *Arabidopsis* (Wang et al. 2007). The SA-mediated growth inhibition may be associated with alternation of GA signaling and responsiveness (Gallego-Giraldo et al. 2011) as well as auxin signaling (Wang et al. 2007). A negative crosstalk between GA signaling and jasmonate signaling has also been reported (Yang et al. 2012). Brassinosteroid (BR) receptor (BRI1)-associated kinase can cooperate with BRI1 for plant growth and with the receptors of microbial-associated molecular patterns for plant immunity. It has been demonstrated that BR-mediated growth antagonizes innate immune response in *Arabidopsis* (Albrecht et al. 2012; Belkadir et al. 2012). BR acts as a virulent factor to suppress defense mechanism against rice root oomycete *Pythium graminicola*, at least in part, through negative crosstalk with SA and GA pathways (De Vleeschauwer et al. 2012). Interestingly, the expression of *OsGA20ox3* is inhibited by *P. graminicola* inoculation, whereas *OsGA20ox3*, the GA-degrading enzyme gene, is induced by *P. graminicola* infection. It was shown that BR, in this case, antagonizes GA-mediated defenses by interfering with GA metabolism, leading to enhanced stabilization of SLR1, the only DELLA protein in rice (De Vleeschauwer et al. 2012). These data highlight the central importance of phytohormone homeostasis in balancing growth and immunity in plants, and our results provide new insights into this balance by demonstrating an important role of *OsGA20ox3* in regulating rice growth and defense.

MATERIALS AND METHODS

Plant growth conditions.

For germination, seeds of the 17S-14 mutant and its WT progenitor (*Oryza sativa* L. japonica, Aichi Asahi) were sterilized and rinsed with sterile water, then imbibed in water at 37°C for 1 day. The germinated seed were transferred to the mixture of peat/vermiculite (3:1, vol/vol) and grown in a greenhouse at ambient temperature (approximately 28°C) under natural sunlight.

Chemical treatments.

Seeds of the 17S-14 mutant and WT were sterilized in water and were germinated at 37°C. The seed-emerged buds were placed on the one-half Murashige-Skoog solid media containing various amounts of S-3307 or GA₃ and grown at 28°C under fluorescent light (14 h of light and 10 h of darkness). The second leaf sheath lengths of seedlings were measured 10 days after treatments, and six plants were evaluated for each treatment.

To examine the effects of GA and its inhibitor on disease resistance, 2-week-old seedlings were sprayed with 20 μM S-3307, 60 μM GA₃, or 0.1% ethanol as the control, six times, at an interval of 4 days. Two days after the last application, seedlings were inoculated with *X. oryzae* pv. *oryzae*.

Construction and screening of a rice subgenomic library.

Genomic DNA of the 17S-14 mutant was digested with *EcoRI* and separated in a 0.8% (wt/vol) low-melting agarose gel. According to the result from parallel Southern blot analysis using another gel, a segment of the gel corresponding to the position of *Hyg* (*Hyg*) phosphotransferase probes was excised out. The DNA purified from the gel was ligated to pBluescript SK II vector. Individual transformants were inoculated into wells of a 384-well microplate and grown in Luria-Bertani medium at 37°C overnight. The bacteria were transferred onto a nylon membrane (Hybond-N⁺; Amersham Pharmacia Biotech, Buckinghamshire, U.K.) and used for screening clones harboring the *Hyg* gene. In total, approximately 2×10^5 clones were screened. DNA labeling and Southern blotting were performed as described by Sakamoto and associates (2001).

Generation of transgenic plants.

The full-length coding sequence of *OsGA20ox3* was isolated by PCR using forward primer OX3gKpnF: 5'-attgtacctgatccatcgacgcgcg-3' and reverse primer OX3gBamHR: 5'-cttgatcctcccatctgtctagtactgtcg-3'. The PCR product was digested with *KpnI* and *BamHI* and inserted into the same sites behind the CaMV35S promoter in a modified pCambia1301 vector.

For making RNAi constructs, fragments of *OsGA20ox3* were amplified by PCR using two forward primers OX3KpnF: 5'-tttggtaccgtgtgtttatcatgcaggagggtgt-3' and OX3HXbaF: 5'-aa taagcttcttagatgtaccaggagtactgcg-3' and paired respectively with a reverse primer OX3SalR: 5'-aaagtcgaccgcgcaggtgcgg-3'. The fragments were constructed to generate self-complementary hairpin RNA after enzyme digestions and ligations. The hairpin structure was placed under the control of the CaMV35S promoter.

For making the promoter-*GUS* fusion construct, the *OsGA20ox3* promoter region of approximately 1,500 bp was obtained by PCR using primers of OX3pF: 5'-ttaaagcttgcgcggtttactgacggattctat-3' and OX3pR: 5'-ttccccgggacgaacaccacggtgcat-3'. The promoter fragment was inserted in frame with the *GUS* gene into a modified pCambia1301 vector to generate *P_{OsGA20ox3}:GUS*.

All DNA constructs were introduced into rice calli of Aichi Asahi by *Agrobacterium*-mediated transformation. Overexpression and RNAi lines in the T₂ generation and promoter lines in the T₁ generation were screened by planting seeds on one-half Murashige-Skoog medium (containing 0.6% agar) supplemented with Hyg at 30 µg/liter, and antibiotic-resistant seedlings were used for experiments.

RT-PCR and real-time PCR.

DNase-treated total RNA samples were reverse transcribed and the cDNA products were used for PCR amplifications. The primers were shown in Supplementary Table S1 and an *OsActin* gene was used as an internal standard for RT-PCR analysis. The PCR products were separated by electrophoresis on a 1.2% (wt/vol) agarose gel, and the DNA bands were visualized using the Alphaimager 2200 system (Alpha Innotech Corporation, San Leandro, CA, U.S.A.). Real-time quantitative PCR was performed using approximately 10 ng of cDNA and SYBR Green PCR MasterMix (Takara Biotech, Dalian, China) in a 15-µl reaction volume using a StepOne Quantitative PCR system (Applied Biosystems, Foster, City, CA, U.S.A.) following the manufacturer's protocols.

Quantitative analysis of GA₁ and GA₄.

Endogenous GA extract and quantitative analysis were performed as described previously (Hirano et al. 2008; Oikawa et al. 2004; Zhu et al. 2006). One-week-old seedlings of mutant 17S-14 and WT were harvested and ground to powder in liquid

nitrogen. The powders (approximately 20 g) were extracted with 300 ml of pre-cooled aqueous methanol (80% MeOH [vol/vol]) for 6 h at 4°C. The extract was collected by filtration through a filter paper. The residue and filter paper were re-extracted with 200 ml of 80% MeOH. The combined filtrates were added with 10 ng of deuterated [17.17-²H₂]GA₁ and 100 ng of [17.17-²H₂]GA₄ (purchased from Australian National University) as internal standards and concentrated to approximately 40 ml under vacuum below 35°C. The aqueous solution left was adjusted to pH 3.0 with 6 M HCl and then extracted with ethyl acetate (EtOAc, 50 ml, three times). The combined EtOAc was dried by Na₂SO₄ and filtered. The filtrate was concentrated to dryness and redissolved in 5 ml of MeOH. The solution was pre-purified in a preparative high-performance liquid chromatography system using an ODS-AP SP-120-15, 250-by-10-mm column (Japan Analytical Industry Co., Ltd., Japan). The fractions collected (60% MeOH containing 1% acetic acid) were evaporated by a stream of nitrogen gas and the residue was resuspended in 100 µl of MeOH. The active GA (GA₁ and GA₄) in the solutions were detected by an LC-MS system (Agilent [Santa Clara, CA, U.S.A.] 1100 Liquid Chromatography with a Phenomenex Luna 3 µm C18, 150-by-2-mm column; Bruker Esquire 6000 mass spectrograph [Billerica, MA, U.S.A.]). The mobile phase was MeOH (containing 0.1% formic acid) with a linear gradient from 30 to 100%. The linear gradient elution program for GA₁ was as follows: 30% MeOH for 5 min, from 30 to 70% MeOH in 20 min, from 70 to 100% MeOH in 5 min, and column washing for 10 min. The linear gradient elution program for GA₄ was as follows: 40% MeOH for 5 min, from 40 to 100% MeOH in 30 min, and 100% MeOH for 10 min. The retention times of GA₁ and GA₄ were approximately 15.8 and 27.3 min, respectively. The characteristic ions were monitored as follows: 329, 303, 285, 241, 259, 301, and 273 for GA₁; 331, 305, 287, 243, 261, 303, and 275 for [17.17-²H₂]GA₁; 313, 287, 243, 269, 225, 285, and 257 for GA₄; and 315, 289, 245, 271, 227, 287, and 259 for [17.17-²H₂]GA₄. The quantity of GA₁ and GA₄ in the original extracts was calculated by the abundance ratio of characteristic ion pairs: 329/331 for GA₁ and [17.17-²H₂]GA₁, and 313/315 for GA₄ and [17.17-²H₂]GA₄, respectively. The other ions were monitored to confirm the identification of the compounds analyzed.

Histochemical GUS staining.

Various organs of transgenic lines of the T₁ generation were prepared for histochemical GUS staining, and WT plants (Aichi Asahi) were used as controls. Samples were incubated in GUS staining buffer (50 mM sodium phosphate [pH 7.0], 10 mM EDTA-Na₂, 20% methanol, 0.1% Triton X-100, and X-Gluc at 0.5 mg/ml) at 37°C for 3 h, after vacuum infiltration for 5 min. Chlorophyll was cleared from the plant tissues by immersing the samples in 70% ethanol. Three independent experiments were performed and more than 10 *P_{OsGA20ox3}:GUS* transgenic plants were observed.

Pathogen growth and inoculation.

Inoculation of rice plants with *M. oryzae* was conducted in a controlled growth chamber as described by Peng and Shishiyama (1988). To examine disease resistance, the virulent strain MS220 was used. Spores were harvested and suspended. Three-week-old plants were inoculated with spore suspensions of *M. oryzae* at 2×10^5 conidia/ml (containing 0.01% silwet) by spray. The plants treated were kept in darkness for 48 h at 28°C and then in a greenhouse under natural sunlight with 95% humidity. After 7 days, the number of lesions was counted on the third leaves of the rice plants and at least eight leaves were evaluated. Leaves of some of these plants were also analyzed for gene expression.

X. oryzae pv. *oryzae* race 6 (strain PX099) was grown in potato dextrose agar medium at 28°C for 3 days and collected by centrifugation. The bacteria were suspended in 10 mM MgCl₂ at a concentration of 10⁸ CFU/ml for inoculation. Six-week-old plants of the 17S-14 mutant, WT, and transgenics were inoculated with PX099 using the leaf-clipping method and grown under natural sunlight in a greenhouse at approximately 28°C. Lesion length was recorded at 12 days post inoculation.

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