**OsGSR1** is involved in crosstalk between gibberellins and brassinosteroids in rice

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Summary

Gibberellins (GAs) and brassinosteroids (BRs), two growth-promoting phytohormones, regulate many common physiological processes. Their interactions at the molecular level remain unclear. Here, we demonstrate that **OsGSR1**, a member of the GAST (GA-stimulated transcript) gene family, is induced by GA and repressed by BR. RNA interference (RNAi) transgenic rice plants with reduced **OsGSR1** expression show phenotypes similar to plants deficient in BR, including short primary roots, erect leaves and reduced fertility. The **OsGSR1** RNAi transgenic rice shows a reduced level of endogenous BR, and the dwarf phenotype could be rescued by the application of brassinolide. The yeast two-hybrid assay revealed that **OsGSR1** interacts with DIM/DWF1, an enzyme that catalyzes the conversion from 24-methylenecholesterol to campesterol in BR biosynthesis. These results suggest that **OsGSR1** activates BR synthesis by directly regulating a BR biosynthetic enzyme at the post-translational level. Furthermore, **OsGSR1** RNAi plants show a reduced sensitivity to GA treatment, an increased expression of the GA biosynthetic gene **OsGA20ox2**, which is feedback inhibited by GA signaling, and an elevated level of endogenous GA: together, these suggest that **OsGSR1** is a positive regulator of GA signaling. These results demonstrate that **OsGSR1** plays important roles in both BR and GA pathways, and also mediates an interaction between the two signaling pathways.

**Keywords:** **OsGSR1**, rice, phytohormone, gibberellins, brassinosteroids, crosstalk.

Introduction

Gibberellins (GAs) and brassinosteroids (BRs) are two important plant hormones that induce similar cellular and developmental responses, including cell elongation, seed germination and flowering (Inada et al., 2000; Steber and McCourt, 2001; Yang et al., 2003; Mussig, 2005). Much progress has been made in understanding the signaling pathways of GA and BR. GA is perceived by GID1, a soluble receptor that mediates GA signaling in rice (Ueguchi-Tanaka et al., 2005). DELLA proteins play a negative role in the control of the GA signaling pathway. The rice DELLA protein, SLR1, directly interacts with GID1 in a GA-dependent manner (Ikeda et al., 2001; Ueguchi-Tanaka et al., 2007), and the interaction leads to the degradation of the DELLA protein by the 26S proteasome (Fu et al., 2002; McGinnis et al., 2003; Sasaki et al., 2003). BRs are a large family of plant steroidal hormones, with brassinolide being the most biologically active C₂₈ BR. Extensive studies using Arabidopsis as a model plant revealed a near complete BR signal transduction pathway, which includes a pair of cell-surface receptor-like kinases, BR1 and BAK1, the cytosolic kinase BIN2, and the nuclear transcriptional factors BZR1 and BES1/BZR2 (Gendron and Wang, 2007). Studies of rice have demonstrated conserved functions of OsBR1 and OsBZR1 in BR signaling (Yamamuro et al., 2000; Bai et al., 2007). However,
little is known about how BRs link with other plant hormones at the molecular level to regulate plant growth.

Previous physiological investigations suggested that GA and BR act independently in promoting plant growth. For example, in tobacco, GA and BR appear to promote seed germination through distinct pathways and mechanisms (Leubner-Metzger, 2001). However, as a deficiency of, or insensitivity to, either hormone results in similar phenotypes, such as dwarfism, reduced seed germination and delayed flowering, the two pathways may interact with each other to achieve optimal growth responses. In support of this notion, genes regulated by both hormones have been identified. A study of a brassinolide (BL)-insensitive mutant showed that both GAS1, a GA-responsive gene, and GAS5, a GA-repressible gene, are antagonistically regulated by GA and BL (Bouquin et al., 2001). The analysis of 4000 expressed sequence tags (ESTs) in rice treated with GA and BR demonstrated some specific genes coordinately regulated by GA and BR (Yang and Komatsu, 2004; Yang et al., 2004). However, the interaction between the GA and BR pathways remains unclear at the molecular level.

The GA-stimulated transcript (GAST) family members share a conserved structural feature: a conserved C-terminal domain consisting of the last 60 amino acid residues with 12 cysteines. This GA-regulated cysteine-rich protein family has been studied in Arabidopsis thaliana, Gerbera hybrida, Petunia hybrida and Solanum tuberosum (Taylor and Scheuring, 1994; Herzog et al., 1995; Kottilainen et al., 1999; Segura et al., 1999; Ben-Nissan et al., 2004). GIP1, the petunia homologue of tomato GAST1, promotes corolla and stem elongation (Ben-Nissan and Weiss, 1996). In contrast, overexpression of GEG in G. hybrida reduces cell elongation in floral organs (Kottilainen et al., 1999). In petunia, GIP1 and GIP2 function in cell elongation, and RNA interference (RNAi) transgenic plants of GIP2 showed reduced stem elongation (Ben-Nissan et al., 2004). Consistently, transgenes of GIP2 driven by the CaMV 35S promoter enhanced stem and corolla elongation, which was suggested to result from a decreased level of reactive oxygen species (Wigoda et al., 2006). These studies suggest that the GAST family genes play an important role in regulating plant development.

Until now, a coordinative mechanism between GA and BR signaling at the molecular level has been unknown. In this study, we demonstrated that the GA-induced OsGSR1 (GAST family gene in rice) was repressed by BR treatment. Reducing OsGSR1 expression by RNAi in transgenic rice caused phenotypes similar to BR-defective mutants, and OsGSR1 interacts with the BR biosynthetic enzyme DIM/DWF1. OsGSR1 RNAi rice has higher levels of endogenous GA, and is less sensitive to GA than wild-type rice. Our results indicate that OsGSR1 is a positive regulator of GA response, and regulates BR biosynthesis, thus providing a link between the GA and BR pathways.

**Results**

OsGSR1 is a GA-responsive GAST family gene in rice

A microarray study showed that the expression of the EST y656d05 was increased by approximately twofold upon treatment with GA3 (Figure 1a) (Wang et al., 2005). Sequence analysis of GAST family proteins shows their common structural features: a putative short peptide, a highly divergent hydrophilic region and a conserved C-terminal domain with 12 cysteine residues in conserved positions (Herzog et al., 1995; Aubert et al., 1998). Based on these characteristics, the gene corresponding to EST y656d05 in rice was designated as OsGSR1 (GAST family gene in rice 1) (GenBank AY604180) (Figure S1). Figure 1b shows the phylogenetic tree of the GAST family proteins in rice and Arabidopsis. OsGSR1 is most closely related to its Arabidopsis homolog AtGASA4, with 48% amino acid sequence identity.

A Southern blot detected only one band in rice, which suggested the presence of one copy of OsGSR1 in the genome (Figure 1c). RT-PCR results showed OsGSR1 expression enhanced by GA3 treatment, with peak OsGSR1 expression detected 4 h after treatment with GA (Figure 2a). Consistent with GA induction, Paclobutrazol, an inhibitor of
GA₃ biosynthesis, repressed the OsGSR1 expression in a dose-dependent manner (Figure 2b). OsGSR1 was expressed in seedlings, roots, shoots, spikes and anthers, but not in mature leaves (Figure 2c). RNA in situ hybridization detected OsGSR1 expression in shoot apical meristems (SAMs), young leaves and adventitious root primordia, and also in the cell division zone of primary roots (Figure 3). Therefore, OsGSR1 is expressed prominently in young and actively growing organs. To determine the subcellular localization of OsGSR1, the OsGSR1:GFP fusion protein as well as GFP alone, as a control, were monitored in cells. As shown in Figure 4, similar to the localization pattern of the GFP control (Figure 4a,b), the OsGSR1:GFP fusion protein was localized in the plasma membrane, cytoplasm and nucleus. Its expression pattern may be related to its function in development regulation.

OsGSR1 RNAi transgenic plants exhibit GA- and BL-related phenotypes

To study the biological role of OsGSR1 in rice, we obtained three knock-down transgenic lines by an RNAi approach (Figure 5a). Using RT-PCR and northern blot, we could not detect OsGSR1 RNA in OsGSR1 RNAi lines 4 or 5, as compared with the wild type (Figures 5b and 6a). Real-time PCR analysis revealed a reduction of OsGSR1 RNA level to 15% that of wild-type plants in transgenic line 4 (Table 1), whereas the other rice GAST homolog genes OsGASR1 and OsGASR2 did not show any significant change in expression patterns (Figure 5c). Compared with the wild type, the three OsGSR1 RNAi transgenic lines showed shorter shoots and primary roots (P < 0.01), and longer coleoptiles (P < 0.05; Figure 6a) (Table 2), and the severity of phenotypes in the different lines correlated with their decreased level of OsGSR1. The cells in the mature zone of primary roots in transgenic line 4 were much shorter than those in the wild type (Figure 6b), which suggests that the short root length of OsGSR1 RNAi plants results from shorter cells, and not from a reduction in cell number.

Adult OsGSR1 RNAi transgenic rice exhibited erect leaves and dwarf phenotypes (Figure 6c). Every internode of

Figure 2. Expression pattern of OsGSR1 in rice.
(a) Expression of OsGSR1 in wild-type rice treated with 5 × 10⁻⁵ M GA₃ for the times shown.
(b) Expression of OsGSR1 in wild-type seedlings treated with paclobutrazol (PAC).
(c) Expression of OsGSR1 in wild-type rice organs detected by RT-PCR.

Figure 3. RNA in situ hybridization.
(a) Shoot apical meristem (SAM) hybridized with sense probe as control.
(b) Signal in SAM with antisense probe.
(c) Enlargement of the panel in (b).
(d) Adventitious root meristem hybridized with sense probe as a control.
(e) Signal in adventitious root meristem.
(f) Enlargement of the panel in (e).
(g) Primary root hybridized with sense probe as a control.
(h) Signal in the primary root. Scale bars: 50 μm (c, e and f); 100 μm (a, b, d, g and h).
OsGSR1 RNAi plants was shorter than that of the wild type (Figure 6d). At the ripe stage, the branches of rachis of OsGSR1 RNAi plants were shorter than those of the wild type, and the fertility was also reduced (Figure 6d).

**OsGSR1 is required for normal GA response**

Three GA dose-dependent experiments were performed to test how OsGSR1 RNAi rice responds to exogenous GA. First, GA3 increases the elongation of the second leaf sheath, a response that was lower in RNAi rice than in the wild type (Figure 7a). Second, GA induces α-amylase activity in seeds.

**Table 1**: Expression of OsGA20ox2, SLR1 and OsGSR1 by real-time PCR analysis

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<td>Ostubulin</td>
<td>1.000</td>
<td>1.000</td>
<td>1.000</td>
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<tr>
<td>OsGA20ox2</td>
<td>0.279</td>
<td>5.46</td>
<td>3.140</td>
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<tr>
<td>SLR1</td>
<td>0.980</td>
<td>3.10</td>
<td>5.210</td>
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<tr>
<td>OsGSR1</td>
<td>1.640</td>
<td>0.62</td>
<td>0.149</td>
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CK, wild-type plants; GA$_3$, wild-type plants treated with GA$_3$; BL, wild-type plants treated with brassinolide (BL); Line 4, seedlings of OsGSR1 RNAi transgenic plants line 4.
In wild-type rice, α-amylase activity was induced at 10^{-9} M GA3, whereas in OsGSR1 RNAi rice, a similar response was induced by 10^{-8} M GA3. The α-amylase activity in the transgenic plants was lower than that of the wild type at up to 10^{-6} M GA3 (Figure 7b), which suggests a reduced GA response in OsGSR1 RNAi plants. Third, the effect of exogenous GA on internode elongation was measured. The internode was elongated by treatment with 10^{-6} M GA3 in the wild type, whereas no change in internode elongation occurred under the same conditions in OsGSR1 RNAi rice (Figure 7c).

To determine whether the GA response phenotypes result from reduced GA levels, or from reduced GA signaling, we measured the levels of endogenous GA and expression of GA biosynthetic genes in the OsGSR RNAi plants. As shown in Table 3, the endogenous GA4 level in OsGSR1 RNAi plants was higher than that in wild-type rice. Consistent with an increased GA level, the expression of OsGA20ox2 and SLR1
was increased in OsGSR1 RNAi rice line 4 (Table 1). Because OsGA20ox2 expression and GA biosynthesis are feedback inhibited by GA signaling, the phenotypes of reduced GA response and increased GA level suggest a primary defect of GA signaling in OsGSR1 RNAi plants, which leads to the reduced feedback inhibition of GA biosynthesis.

OsGSR1 is involved in the regulation of BR biosynthesis

In contrast to GA induction, BR treatment reduced OsGSR1 expression (Figure 8a). Real-time RT-PCR revealed that both BL treatment and RNAi suppression of OsGSR1 enhanced the expression of OsGA20ox2 and SLR1 (Table 1), which suggests a role of OsGSR1 in BR action. To further test the effects of OsGSR1 RNAi on the rice growth response to BR, transgenic rice was treated with 24-epibrassinolide (eBL). Lamina joint bending is one of the most sensitive responses to BR in rice (Wada et al., 1981). Without BR treatment, the degree of bending of joints between the leaf sheath and blade was smaller in OsGSR1 RNAi transgenic rice than in the wild type, but was greater than that in the wild type after treatment with a low concentration of 24-eBL (Figure 8b). At 0.5 ng ml$^{-1}$ 24-eBL, the bending degree in line 4 was about 90°, as compared with 77° in wild-type rice. The lamina joint bending of OsGSR1 RNAi transgenic rice was the same as that of the BR-deficient mutants d2 and d11 upon treatment with BL (Hong et al., 2003; Tanabe et al., 2005). The dwarf phenotype of OsGSR1 RNAi transgenic rice was restored by exogenous treatment with 10$^{-6}$ M 24-eBL at the seedling stage (Figure 8c). In contrast, wild-type rice showed no response to this treatment. The rescue of dwarf and erect-leaf phenotypes by BR treatment suggests that the OsGSR1 RNAi transgenic rice is deficient in BRs. Indeed, measurement of endogenous BRs in the OsGSR1 RNAi plants revealed reduced levels of campesterol (CR) and campestanol (CN).

We monitored the expression of OsBRI1 and OsDWARF, which encode the BR receptor and a BR biosynthetic enzyme. Their expression is feedback inhibited by BR signaling. OsGSR1 RNAi transgenic plants showed higher OsBRI1 and OsDWARF expression than the wild-type plants (Figure 8d), which is consistent with reduced BR signaling resulting from the decreased level of endogenous BR in OsGSR1 RNAi transgenic rice.

Table 3 Endogenous level of GA$_4$ measured by gas chromatography-mass spectrometry

<table>
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<tr>
<th>Wild type (ng g$^{-1}$ FW fresh weight)</th>
<th>OsGSR1 transgenic Line 4 (ng g$^{-1}$ FW fresh weight)</th>
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<tr>
<td>Endogenous level of GA$_4$</td>
<td>4.23 ± 1.03</td>
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<td>24.18 ± 2.14</td>
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Samples were measured in three independent experiments.
OsGSR1 interacts with DIM/DWF1 in vitro and in vivo

To further determine the function of OsGSR1, a yeast two-hybrid system was used to screen proteins interacting with OsGSR1. The full-length OsGSR1 was used as the bait. Positive interaction of OsGSR1 was verified based on both survival on a limited medium (SD/-His/-Ade/-Trp/-Leu) and expression of the β-galactosidase reporter gene (Figure 9a). Seven positive clones were identified among the approximately $2.5 \times 10^6$ cDNA clones. Three of them corresponded to a full-length cDNA of 1686 bp that encodes the DIM/DWF1 gene, an enzyme that catalyzes the conversion of 24-methylenecholesterol (24-MC) to campesterol (CR), and were involved in BR biosynthesis (Hong et al., 2005). In a pull-down assay, the GST-OsGSR1 protein was immobilized to glutathione-sepharose beads, and His-tagged DWF1 protein was incubated with the beads. The DWF1-His protein co-purified with GST-OsGSR1, which indicated a direct interaction between OsGSR1 and DWF1 in vitro (Figure 9b).

The in vivo interaction between OsGSR1 and DIM/DWF1 was analyzed by the bimolecular fluorescence complementation system (BiFC) (Bracha-Drori et al., 2004; Walter et al., 2004). In this system, the yellow fluorescent protein (YFP) was split into N-terminal (YN) and C-terminal (YC) halves; fluorescence was observed when two proteins fused to each YFP half interacted with each other. Strong YFP fluorescence could be observed when YN-OsGSR1 and YC-DIM/DWF1 were co-expressed in tobacco leaf epidermal cells (Figure 9c). In contrast, no YFP signal was observed when YN-OsGSR1 and no-fusion YC, as the control, were co-transformed. These results demonstrate that OsGSR1 interacts with DIM/DWF1 in vivo.

DIM/DWF1 catalyzes the conversion of 24-MC to CR in rice and Arabidopsis (Klahre et al., 1998; Hong et al., 2005). The levels of endogenous 24-MC, CR and CN were analyzed by gas chromatography-mass spectrometry (GC-MS). In OsGSR1 RNAi transgenic line 4, the level of 24-MC was three times that of the wild type, whereas the level of CR was about 25% that of the wild type; the level of CN was also decreased in the OsGSR1 RNAi plants (Figure 9d). This altered BR level is consistent with reduced DIM/DWF1 activity in the OsGSR1 RNAi transgenic rice. These results suggest that OsGSR1 positively regulates the activity of DIM/DWF1.

Discussion

OsGSR1 is antagonistically regulated by GA$_3$ and BL

The GAST family represents genes that are all structurally related to the original GA-regulated GAST1 gene from
tomato. Besides GA, some other plant hormones regulate the expression of GAST family genes. The transcript level of RS1 was induced by both GA and N-acetyl aspartate in tomato (Taylor and Scheuring, 1994). ABA partially inhibits the GA-induced GAST1 RNA abundance in tomato (Shi and Olszewski, 1998). SNAKIN was upregulated by ABA and wounding in potato (Berrocal-Lobo et al., 2002).

In our study, GA3 treatment resulted in a significant increase in the level of OsGSR1 within 4 h (Figure 2a). BL treatment decreased OsGSR1 expression in wild-type rice (Figure 8a). Therefore, OsGSR1 is a GAST rice gene that is upregulated by GA3 and downregulated by BL. This expression pattern is the same as in the OsGSR1 homolog GAS1 (Bouquin et al., 2001). RNA in situ hybridization detected OsGSR1 expression primarily in all meristematic regions (Figure 3). OsGSR1 may play an important role in these regions, and the following evidence shows that OsGSR1 in SAMs modulates the internode elongation of rice. Moreover, the antagonistic effects of GA and BR suggest the involvement of OsGSR1 in both signaling pathways.

OsGSR1 is involved in crosstalk between GAs and BRs in rice

Bioactive GAs are diterpene plant hormones involved in regulating many physiological processes. Changes in both GA concentration and signal transduction influence plant growth. The characterization of loss-of-function and GA-unresponsive dwarf mutants has identified several positive regulators of GA signaling. The dwarf1 (d1) (Ueguchi-Tanaka et al., 2000) and GA-insensitive dwarf2 (gid2) (Sasaki et al., 2003) mutants in rice have a semidwarf phenotype that cannot be rescued by GA treatment. In our GA-response experiment, OsGSR1 RNAi transgenic rice plants were less sensitive to GA3 than wild-type plants, and OsGSR1 RNAi transgenic rice showed reduced α-amylase activity and elevated GA content, similar to the d1 mutant. These results indicate that RNAi suppression of OsGSR1 influences the GA signaling pathway, and that OsGSR1 acts as a positive regulator of GA signal transduction.

The GA homeostasis is achieved by the feedback mechanism, through collaboration between GA biosynthesis and response pathways. The expression of OsGA20ox2, a GA 20-
oxidase gene, is feedback regulated to balance the level of GA (Spielmeyer et al., 2002). SLR1, the rice GAI or RGA homolog, is a negative regulator of GA signaling. Recently, the global analysis of DELLA direct targets found that GA20ox2 may be a direct DELLA target in Arabidopsis (Zentella et al., 2007). In OsGSR1 RNAi transgenic rice, the expression level of OsGA20ox2 and SLR1 is opposite to that of GA-treated wild-type rice (Table 1). The reduced GA response in OsGSR1 RNAi plants could result from the upregulation of SLR1, and OsGSR1 might positively regulate GA response by downregulating SLR1 expression. As is known, GAs induced the degradation of the SLR1 protein through the SCF<sup>GA20ox2</sup>-proteasome pathway in rice (Sasaki et al., 2007). Our data showed that transcriptional expression of SLR1 was not induced by treatment with GA in wild-type rice (Table 1), which was consistent with a previous study in rice (Itoh et al., 2002). The knock-down of GSR1 could decrease the biosynthesis of GA and elevate the expression of SLR1 at a transcriptional level. These results suggested that OsGSR1 regulates the expression of SLR1 not exclusively via GA. Some other pathways may also be involved. There may be alternative pathway(s) for GA signaling, also discussed in other studies (Nakajima et al., 1997; Ueguchi-Tanaka et al., 2000). We demonstrated that OsGSR1 participated in the BR signaling pathway by directly interacting with DIM/DWF1, so BR signaling may be involved in regulating the expression of SLR1.

OsGSR1 is involved in the regulation of BR biosynthesis

Previous study showed that BRs did not undergo long-distance transport, and they acted at the site of synthesis. Thus, cells or tissue alone must monitor BR biosynthesis to achieve optimal development. This mechanism has been studied in detail at the molecular level. BZR1, as a transcriptional repressor of BR signaling, binds directly to the promoters of the feedback-regulated BR biosynthetic gene CPD (He et al., 2005). Several other factors must influence BR biosynthesis. Here, we describe how OsGSR1 participates in BR biosynthesis.

First, BR-insensitive and -deficient mutants show some unique characteristics in rice. The dwarf phenotype and erect leaves of OsGSR1 RNAi transgenic rice (Figures 8c and 8d) led us to speculate that OsGSR1 is involved in either biosynthesis or signal transduction of BRs. Lamina inclination is generally accepted as a sensitive bioassay for BR in rice. Phenotypic analyses of rice BR-deficient mutants demonstrated that the degree of lamina joint bending is very sensitive to exogenous BR (Hong et al., 2003; Tanabe et al., 2005). In OsGSR1 RNAi plants, we observed the increased sensitivity to BR in the lamina joint bending test (Figure 8b).

Second, OsGSR1 expression is repressed by BL at the transcriptional level (Figure 8a), which is similar to the expression of BR biosynthesis genes such as D11, D2 and OsDWARF, which are feedback regulated by BL. Furthermore, the BR-insensitive mutant d61 cannot respond to exogenous BL, but d2 and d11 can respond to exogenous BL, and BR rescues their dwarf phenotype. The dwarf phenotype of OsGSR1 RNAi transgenic plants can be restored by treatment with exogenous 24-eBL (Figure 8c). Thus, OsGSR1 RNAi transgenic rice can respond to exogenous BR, and may be defective in endogenous BR biosynthesis. Indeed, OsGSR1 RNAi plants showed a reduced level of endogenous BRs. However, the expression of OsDWARF was significantly increased in transgenic rice (Figure 8d), which is likely to be a consequence of reduced feedback inhibition rather than a cause of altered BR levels. Instead of OsGSR1 regulating transcription of BR biosynthetic genes, its primary action on BR levels appears to result from its direct interaction with the BR biosynthetic enzyme DIM/DWF1. Finally, evidence from both yeast two-hybrid and in vivo BiFC assays supports the theory that OsGSR1 interacts with DIM/DWF1 (Figure 9a,c). The tissue-specific expression pattern of OsGSR1 (Figures 2c and 3) is similar temporally and spatially to that of DIM/DWF1 (Hong et al., 2005). As already known, sterol is mainly synthesized in the endoplasmic reticulum (ER) and in peroxisomes in animal cells. Similarly, in plant cells, BR biosynthesis occurred in some speckled structures within the cytoplasm. For example, DIM/DWF1 is a membrane-associated protein localized in speckled structures within the cytoplasm in Arabidopsis (Klahre et al., 1998), which agrees with their interaction within the cytoplasm in tobacco leaf epidermal cells, as seen in the BiFC assay (Figure 9c). There are many scaffold proteins involved in the traffic between the cytosol and the membrane. This suggests that OsGSR1 may participate in the traffic between the cytosol and the membrane during BR biosynthesis and actions.

Together, these results provide evidence that OsGAS1 is involved in the regulation of BR biosynthesis at the post-translational level. BRs are considered to be essential plant hormones, the endogenous levels of which must be properly maintained for normal growth and development. Detailed study of the biosynthesis of BL reveals that BR biosynthetic pathways in rice are highly networked. The early and late C-6 oxidation pathways connect at many steps, and link to the early C-22 oxidation pathway (Fujioka and Yokota, 2003). In Arabidopsis and rice, DIM/DWF1 protein catalyzes the conversion of 24-MC to CR in the synthesis of bioactive BRs. The reduced conversion of 24-MC → CR → CN in OsGSR1 RNAi rice (Figure 9d) suggests that OsGSR1 binding to DIM/DWF1 activates its enzyme activity. OsGSR1 is a positive regulator of the BR biosynthetic pathway, with roles in BR-induced growth response.
OsGSR1 mediates the crosstalk between GA and BR signaling in rice

Multiple hormones often participate in the same biological process. Therefore, the way in which different hormones cooperatively regulate a developmental process is an important question that has been studied extensively at physiological and molecular levels. The antagonistic effects of GA and ABA on seed germination have been well documented. DELLA restricts GA-promoted processes by modulating the ABA pathway through its target XERICO (Zentella et al., 2007). Elongation and gravitropic responses of Arabidopsis roots are regulated by both BL and indole acetic acid, and the regulation is achieved in part by modulating biosynthetic pathways (Kim et al., 2007b).

Although early investigation indicated that GA and BR act independently in promoting plant growth and development (Leubner-Metzger, 2001), there is increasing evidence for crosstalk between the two hormones in regulating plant development (Bouquin et al., 2001; Yang and Komatsu, 2004; Jager et al., 2005). Recent research indicated that the rice SPINDLY gene acts as a negative regulator of GA signaling, and modulates BR biosynthesis (Shimada et al., 2006). Our results demonstrate that in OsGSR1 RNAi rice, the expression pattern of OsGA20ox2 and SLR1 was similar to that of wild-type plants treated with BL (Table 1). OsGSR1 knock-down and BL treatment have similar effects on the expression of these two genes from the GA signal pathway, which indicates that there are interactions between the GA and BR signal pathways. Consistently, OsGSR1 RNAi plants show the phenotypes of short seminal roots, dwarfism and short grains. Dwarfism, especially, is an important agronomic character, and molecular genetic approaches have revealed that plant dwarfism is often caused by defects in the biosynthesis and perception of GA and BR. The elongated uppermost internode (EUI)-amylase and internode elongation (Ueguchi-Tanaka et al., 2000). Elongated uppermost internode (EUI) functions as a new GA-deactivating enzyme during the growth of internodes during its phase change. The rice dwarf mutant d1 affects a part of the GA signaling pathway, including the induction of α-amylase and internode elongation (Ueguchi-Tanaka et al., 2000). Elongated uppermost internode (EUI) functions as a new GA-deactivating enzyme during the growth of internodes.

OsGSR1 is a positive regulator of the GA signal. Furthermore, OsGSR1 participates in the GA signaling pathway through regulating the expression of SLR1. Evidence of the interaction between OsGSR1 and DIM/DWF1 suggests that OsGSR1 directly regulates BR biosynthesis. Therefore, from our results, we propose that OsGSR1 acts as a coordinator in the crosstalk between GA and BR signaling, which regulate common developmental processes.

Experimental procedures

Plant materials and Agrobacterium-mediated transformation

Rice (Oryza sativa ssp. japonica cv. Zhonghua 10) seeds were germinated and transferred to the field after 3 weeks.

The open reading frame (ORF) of OsGSR1 was amplified using primers: OsGSR1F (KpnI and SpeI restriction sites were introduced) and OsGSR1R (BanHI and SacI restriction sites were introduced) (Table S1). The PCR products were inserted into the RNAi vector pTCK303 as previously reported (Wang et al., 2004). Rice embryonic calli were induced in media of ND2, and then the OsGSR1 RNAi construct was transformed by Agrobacterium tumefaciens EHA105 (Xu et al., 2005). Transgenic plants of the T0 generation from calli were selected in half-strength MS medium containing 75 mg l−1 hygromycin (Sigma-Aldrich, http://www.sigmaaldrich.com). For phenotype analysis, seeds of the T0 generation were germinated in water for 1 week. Seedlings with positive GUS activity were used as OsGSR1 RNAi transgenic rice, and GUS-negative seedlings were used as the control.

Southern blot analysis

The genomic DNA of transgenic plants and control plants was extracted. DNA (30 μg) was digested by EcoRI and BamHI, respectively. After electrophoresis, DNA was transferred and cross-linked onto a Hybond-N+ membrane (Amersham Biosciences, http://www.amersham.com). The GUS gene (800 bp) amplified from the pCAMBIA1301 vector was labeled with [α-32P]dATP and [α-32P]dCTP (China Isotope Inc., Beijing, China, http://www.china-isotope.com) by PCR. After hybridization, the membrane was exposed to X-ray film (Kodak, http://www.kodak.com) at ~70°C for 1 week. The sequences of the GUS primers are listed in Table S1.

RNA preparation and RT-PCR analysis

Total RNA was extracted with Trizol reagent (Invitrogen, http://www.invitrogen.com). The quality of the total RNA was monitored with the use of the DU 640 Nucleic Acid & Protein Analyzer (Beckman Coulter, http://www.beckmancoulter.com). The detailed information about primer sequences for RT-PCR is listed in Table S1. The RT-PCR results were quantified and standardized by comparison with the level of actin using ofBIO-1D software.

Northern blot analysis

Total RNA was isolated using Trizol reagent (Invitrogen). The concentration of total RNA was monitored with the DU 640 Nucleic Acid & Protein Analyzer. RNA (30 μg) was loaded, and full-length OsGSR1 cDNA was labeled with [α-32P]dATP and [α-32P]dCTP, and then used as a probe. Total RNA stained with ethidium bromide (EtBr) was used as a loading control. OsGSR1 primers used in amplification for northern blot analysis are listed in Table S1.

Subcellular localization analysis of OsGSR1

The cDNA sequence of OsGSR1 was amplified with primers OsGSR1-GF and OsGSR1-GR (Table S1). The PCR products were digested with
XbaI and KpnI, and then ligated into pGFP221 vector, in which the coding sequence of the OsGSR1 gene was fused to the 5’ terminus of the GFP gene in frame, driven by the cauliflower mosaic (CaMV) 35S promoter. The fusion construct as well as the control vector with GFP alone were transformed into the onion epidermis cells by particle bombardment (Varagona et al., 1992). After 24 h of incubation, GFP fluorescence in transformed onion cells was observed under a Nikon fluorescence microscope (http://www.nikon.com).

**RNA in situ hybridization**

We used 14-day-old rice seedlings grown on half-strength MS. A direct repeat of a 150-bp OsGSR1 gene-specific fragment was used as the probe (Table S1). The digoxigenin labeling of cRNA probes and in situ hybridization were performed as described by Xu et al. (2002). Images were captured on a microscope (Zeiss, http://www.zeiss.com).

**Real-time PCR**

Reverse transcription was performed with the TaqMan Reverse Transcription Reagents Kit (Applied Biosystems, http://www.appliedbiosystems.com). The cDNA samples were diluted to 5 and 1.25 ng μl⁻¹. Triplicate quantitative assays were performed for each cDNA dilution using the SYBR Green Master mix (Applied Biosystems) in an ABI 7900 sequence detection system, following the manufacturer’s protocol (Applied Biosystems); the gene-specific primers were designed with PRIMEREXPRESS. The relative quantification method (Delta-delta Ct method (DDCT); Yoshida et al., 2002). Images were captured on a microscope (Zeiss, http://www.zeiss.com).

**GA induction in second leaf sheath elongation, internode elongation and α-amylase activity**

Rice seeds were sterilized by 1%, HgCl₂, and were then washed five times with sterilized water. The seeds were then placed on half-strength MS medium containing various concentrations of GA₃ and were incubated at 30°C under continuous light. After 7 days, the lengths of the second leaf sheaths were measured.

Rice seeds were sown in soil and grown for 2 weeks. The seedlings were then irrigated with water containing 1 × 10⁻₆ M GA₃ once a week. The treatment duration was for 4 weeks. The length of elongated internodes was measured.

Embryoless half seeds were sterilized by 2% NaClO, and were washed five times with sterilized water. The induction and measurement of α-amylase activity was performed as described previously (Ueguchi-Tanaka et al., 2000).

**Yeast two-hybrid assay**

A two-hybrid cDNA library of rice seedlings was constructed according to the Clontech protocols (Clontech, http://www.clontech.com). The titer of the library was determined after amplification. The cDNA encoding the full-length OsGSR1 protein was inserted into the GAL4 DNA binding-domain vector pGBKKT7. The rice cDNA library in the GAL4 activation domain vector pGADT7 was screened, and isolation of the positive clones involved used the MATCHMAKER GAL4 Two-Hybrid System 3 and libraries. The full-length cDNA of DIM/DWF1 was amplified with primers DWF1F and DWF1R (Table S1), and were inserted into the pGADT7 vector.

**Pull-down assay**

To test the interaction between OsGSR1 and DIM/DWF1, OsGSR1 was cloned into the pGEX4T-1 vector as a GST fusion protein, and DWF1 was cloned into the pET28a (+) vector to express the proteins in His-tagged form in Escherichia coli, respectively. Purified DWF1-His protein was mixed with the GST-OsGSR1 fusion protein attached to glutathione-raphase beads, as described previously (Shi et al., 1999). After gentle rotation for 20 h, the beads were centrifuged and washed five times. Samples underwent 12% SDS-PAGE, and were transferred onto polyvinylidene fluoride membranes to detect GST-OsGSR1 and DWF1-His by western blot procedure with anti-GST antibody and anti-His antibody, respectively.

**Measurement of endogenous GA₃**

Shoots (about 100 mg fresh weight) of OsGSR1 RNAi transgenic rice, as well as of wild type, were harvested after growing for 2 weeks from germination. The material disruption was carried out using a vibrating-ball micromill. Then the homogenized tissue was incubated in the 80% acetone for 1 h at room temperature (25°C). After centrifugation, the solvent was removed in a vacuum centrifuge. The dried residue was dissolved in 30 μl methanol. Then, 200 μl diethyl ether was added, followed by agitation in the microcentrifuge. Any particles were removed by centrifugation, and the sample was then applied to a microscale aminopropyl solid-phase extraction cartridge. After being silylated with 45 μl of pyridine and 60 μl of N₂O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) for 30 min at 80°C, quantitative analysis of GA₃ was performed using ²H-labeled GA₃ as an internal standard via an Agilent/LECO gas chromatography-mass spectrometer (Muller et al., 2002; Kim et al., 2007a).

**Measurement of endogenous phytosterols**

Shoots of wild-type and OsGSR1 RNAi transgenic line 4 plants were harvested after growth in a glasshouse for 2 months. An quantity of lyophilized shoots (10–12 g) was extracted and analyzed by gas chromatography-mass spectrometry. The levels of endogenous sterols were determined on the basis of calibration curves constructed from the ratios of the M+ peak area of ²H₂-labeled cholesterol added as an internal standard (Klairhe et al., 1998).

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OsGSR1 is involved in crosstalk between GAs and BRs in rice


