Plant Signaling & Behavior 6:12 1932-1936; December 2011; ©2011 Landes Bioscience

A study of phytohormone biosynthetic gene expression using a circadian clock-related mutant in rice

Hironori Itoh and Takeshi Izawa*

Functional Plant Research Unit; National Institute of Agrobiological Sciences; Tsukuba, Japan

Keywords: circadian clocks, phytohormone, gibberellin 2-oxidase, rice, microarray

Abbreviations: Os, *Oryza sativa*; GI, GIGANTEA; GA, gibberellin; ABA, abscisic acid; JA, jasmonic acid; CK, cytokinin; IAA, indole-3-acetic acid

We have recently isolated a rice circadian clock-related mutant carrying a null mutation in *Os-GIGANTEA*(*GI*) gene, the solo ortholog of Arabidopsis *GI*. Time-course global transcriptome analyses of leaves from wild-type and *osgi* mutant grown in the field have revealed that *Os-GI* affects gene expression of more than half of genes on rice 44k microarray. To better understand the biological significance of circadian clock function in growth and development of rice, we here investigated the gene expression involved in phytohormone biosynthesis. Here we found that mRNA levels of a few major genes encoding GA2-oxidase which can inactivate bioactive gibberellins (GAs) were remarkably increased in *osgi-1* plants. This suggests that *Os-GI* functions to maintain bioactive GA level through the regulation of the GA-deactivating enzyme genes in rice. Consistently, *osgi-1* plants showed semi-dwarf phenotype with reduced internode and leaf sheath elongation.

Molecular genetics using Arabidopsis have revealed that circadian clocks control variety of physiological responses including flowering, hypocotyl elongation and guard cell opening.[1] We have recently identified a rice circadian-clock related mutant, *os-gigantea (osgi)* mutant.[2] *Os-GI* is the solo ortholog of *GIGANTEA* (*GI*) in the Arabidopsis circadian system.[1,3,4] Time-course extensive transcriptome analyses of *osgi-1* leaves grown in the field revealed that *Os-GI* affects gene expression of approximately 75% of genes among 27,201 genes and is required for strong amplitudes and proper phase-setting of global gene expression under natural field conditions.[2] These field-transcriptome data imply that circadian clock in rice affects various physiological traits during growth and development of rice. In fact, a critical deficiency of photoperiodic flowering of *osgi-1* plants has been shown due to a great reduction of mRNA levels of *Hd3a*, a rice florigen gene, and *Ehd1*, an inducer of *Hd3a* under laboratory short-day conditions.[2,5] To deeper understand the molecular function of circadian clock in rice, we are comparing the field-transcriptome data between wild-type (the cultivar Norin 8) and *osgi-1* plants and trying to find out key gene controlled by circadian clock systems. In this study, we analyzed the expression of phytohormone biosynthetic genes since circadian clock modulates hormonal action through the transcriptional regulation of phytohormone-related genes in Arabidopsis.[6]

The transcriptome analyses were performed by using leaves of wild-type and *osgi-1* plants sampled for 1 d (24 h) at 2-h intervals (total 13 time points).[2] At each of time point, eight arrays were used with two replicates at four developmental stages. Because each microarray raw data converted to a logarithm fits to normal distribution, we first normalized all wild-type and *osgi-1* microarray data converted to $\log_2$ using the qspline method, which was implemented in R and included in the Bioconductor package (www.r-project.org/).[7] Mean of all eight normalized array data sets per time point was used for further analysis. In this case, we did not focus on the difference of developmental stages and instead used as eight replicated samples per time point to highlight the *Os-GI* in the diurnal rhythms. We then listed up genes involved in seven phytohormone biosynthetic pathways, gibberellin (GA), abscisic acid (ABA), auxin, brassinosteroid, cytokinin (CK), jasmonic acid (JA) and ethylene for this study (**Table S1**) based on recent publication on the comprehensive transcriptome analysis of phytohormone biosynthesis and signaling genes during rice anther development.[8] Hirano et al. also showed that bioactive GA, free IAA, bioactive CK and ABA were detected in leaf blades, indicating that phytohormone biosynthesis works in rice leaves.[8] To address whether *Os-GI* regulates the expression of phytohormone biosynthetic genes, the mean of the gene expression levels for 24 h and their ratios between wild-type and *osgi-1* plants were calculated (**Fig. 1**). Although most tested genes showed only slight

*Correspondence to: Takeshi Izawa: Email: tizawa@affrc.go.jp
Submitted: 08/09/11; Revised: 09/22/11; Accepted: 09/23/11
DOI: 10.4161/psb.6.12.18207

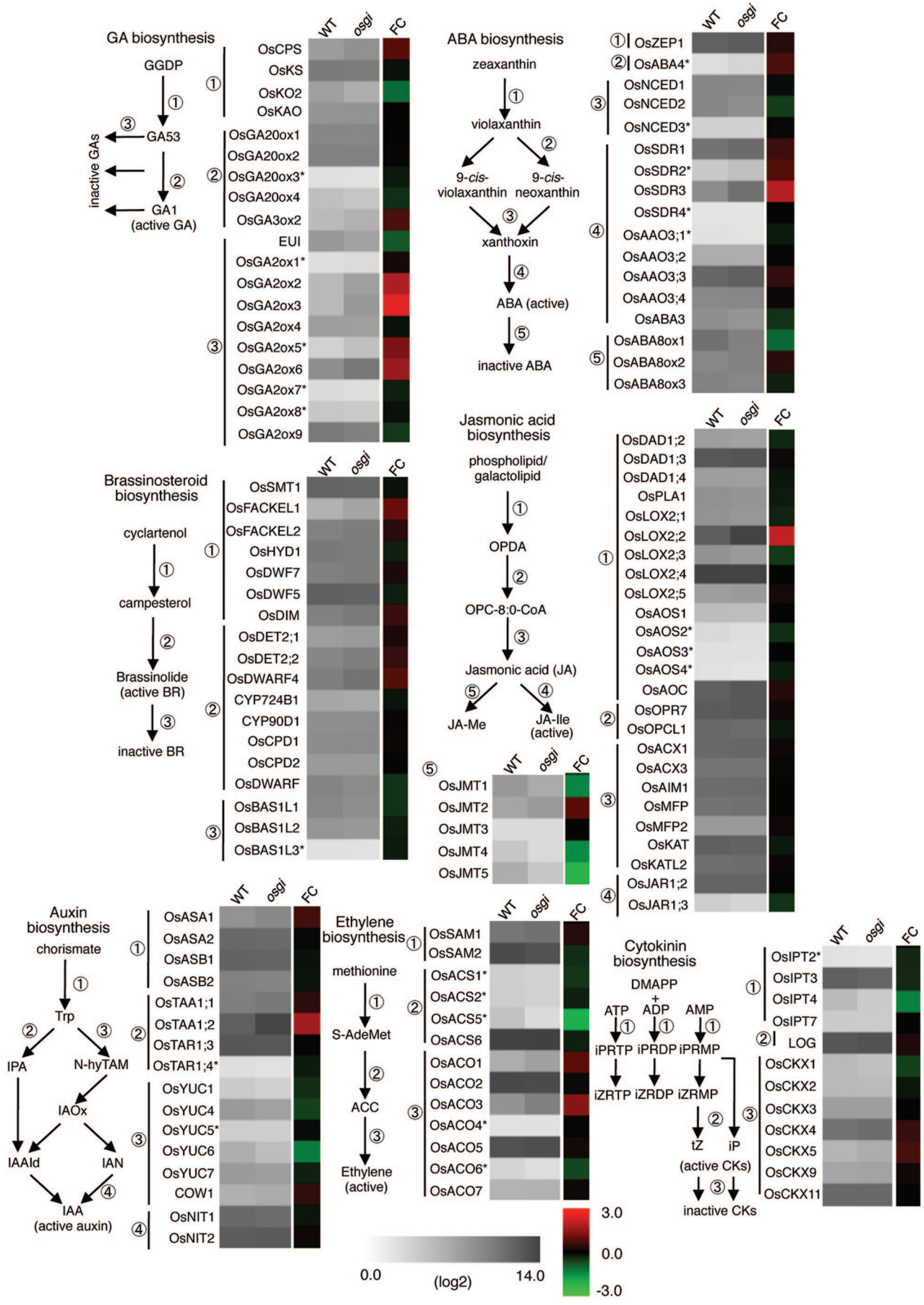
GA biosynthesis
GGDP
GA53
GA1
(active GA)
inactive GAs
OsCPS
OsKS
OsKO2
OsKAO
OsGA20ox1
OsGA20ox2
OsGA20ox3*
OsGA20ox4
OsGA3ox2
EUI
OsGA2ox1*
OsGA2ox2
OsGA2ox3
OsGA2ox4
OsGA2ox5*
OsGA2ox6
OsGA2ox7*
OsGA2ox8*
OsGA2ox9
ABA biosynthesis
zeaxanthin
violaxanthin
9-cis-violaxanthin
9-cis-neoxanthin
xanthoxin
ABA (active)
inactive ABA
OsZEP1
OsABA4*
OsNCED1
OsNCED2
OsNCED3*
OsSDR1
OsSDR2*
OsSDR3
OsSDR4*
OsAAO3;1*
OsAAO3;2
OsAAO3;3
OsAAO3;4
OsABA3
OsABA8ox1
OsABA8ox2
OsABA8ox3
Brassinosteroid biosynthesis
cyclartenol
campesterol
Brassinolide (active BR)
inactive BR
OsSMT1
OsFACKEL1
OsFACKEL2
OsHYD1
OsDWF7
OsDWF5
OsDIM
OsDET2;1
OsDET2;2
OsDWARF4
CYP724B1
CYP90D1
OsCPD1
OsCPD2
OsDWARF
OsBAS1L1
OsBAS1L2
OsBAS1L3*
Jasmonic acid biosynthesis
phospholipid/ galactolipid
OPDA
OPC-8:0-CoA
Jasmonic acid (JA)
JA-Me
JA-Ile (active)
OsJMT1
OsJMT2
OsJMT3
OsJMT4
OsJMT5
OsDAD1;2
OsDAD1;3
OsDAD1;4
OsPLA1
OsLOX2;1
OsLOX2;2
OsLOX2;3
OsLOX2;4
OsLOX2;5
OsAOS1
OsAOS2*
OsAOS3*
OsAOS4*
OsAOC
OsOPR7
OsOPCL1
OsACX1
OsACX3
OsAIM1
OsMFP
OsMFP2
OsKAT
OsKATL2
OsJAR1;2
OsJAR1;3
Auxin biosynthesis
chorismate
Trp
IPA
N-hyTAM
IAOx
IAAld
IAN
IAA (active auxin)
OsASA1
OsASA2
OsASB1
OsASB2
OsTAA1;1
OsTAA1;2
OsTAR1;3
OsTAR1;4*
OsYUC1
OsYUC4
OsYUC5*
OsYUC6
OsYUC7
COW1
OsNIT1
OsNIT2
Ethylene biosynthesis
methionine
S-AdeMet
ACC
Ethylene (active)
OsSAM1
OsSAM2
OsACS1*
OsACS2*
OsACS5*
OsACS6
OsACO1
OsACO2
OsACO3
OsACO4*
OsACO5
OsACO6*
OsACO7
Cytokinin biosynthesis
DMAPP
ATP
ADP
AMP
iPRTP
iPRDP
iPRMP
iZRTP
iZRDP
iZRMP
tZ
iP
(active CKs)
inactive CKs
OsIPT2*
OsIPT3
OsIPT4
OsIPT7
LOG
OsCKX1
OsCKX2
OsCKX3
OsCKX4
OsCKX5
OsCKX9
OsCKX11
WT
osgi
FC
3.0
0.0
-3.0
0.0
(log2)
14.0

Figure 1 (See previous page). Comparison of gene expression levels of phytohormone biosynthetic genes. Average mRNA levels during 24 h in the wild-type (WT) and *osgi-1* plants are indicated as single color gradient. Their ratios (FC; fold change) between WT and *osgi-1* are indicated as different color gradient. Red and green colors indicate higher and lower expression in *osgi-1* mutant, respectively. A simplified view of each phytohormone biosynthetic pathway and genes involved in the indicated step are described in left sides of the expression data. Asterisks on right shoulder of gene name indicate that peaked expression level was lower than 2^6. Color gradient display was drawn by means of software, MeV_4_7.[25]

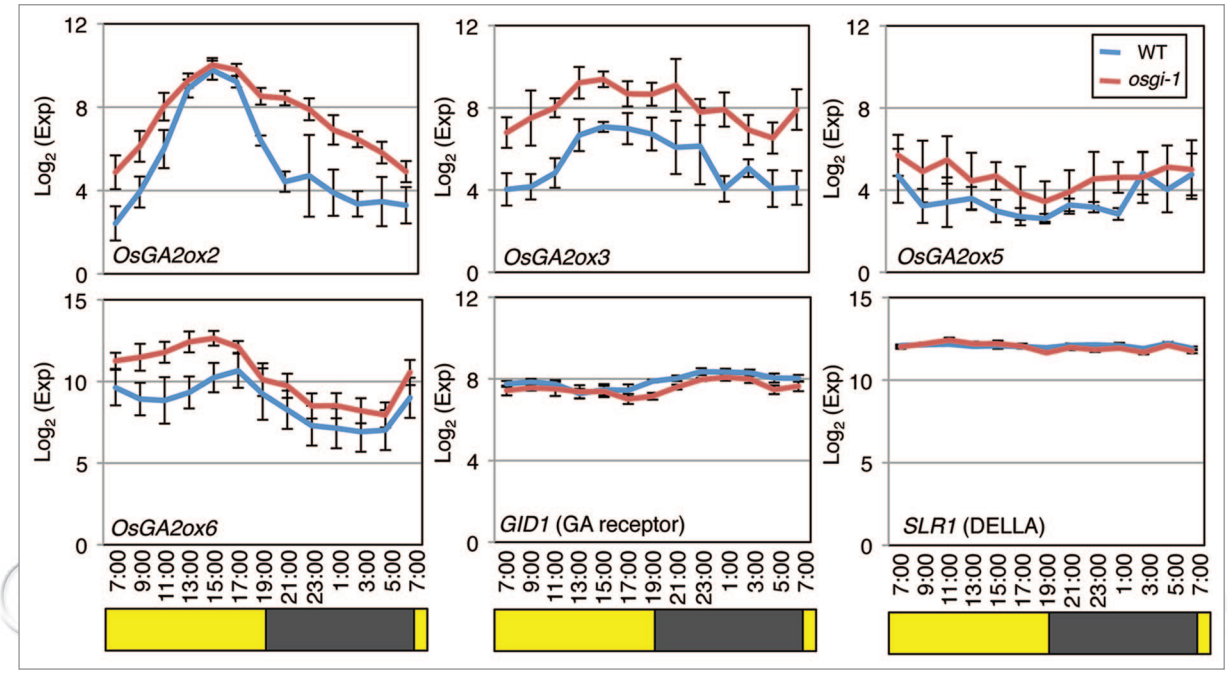

Figure 2. Diurnal expression profiles of the selected *OsGA2ox* genes, *GID1* and *SLR1*. Average calibrated data were expressed as $\log_2$ scale. Data are represented as means ± SE (n = 8). Yellow and black boxes below sampling time indicate day and night periods are referred from the estimated photoperiods described in the recently published paper.[2]

differences between wild-type and *osgi-1* plants (less than 2-fold), however, some were remarkably affected in *osgi-1* (bright red or green boxes indicated in **Fig. 1**). Among them, we found that some members in gene families are consistently changed in *osgi-1*. In GA biosynthesis, four of nine *OsGA2ox* genes were upregulated in *osgi-1* (3- to 5.8-fold increase). Three of five *OsJMT* genes in JA biosynthesis were downregulated in *osgi-1* (2.6- to 4.5-fold reduction). In ABA biosynthesis, two *OsSDR* genes in *osgi-1* were increased with lesser extent (1.5- to 3.9-fold increase). In addition, the expression of *OsKO2* gene in early step of GA biosynthesis was reduced in *osgi-1* plants. In auxin biosynthesis, *OsTAA1;2* is highly expressed in *osgi-1*. Although genetic studies in Arabidopsis have demonstrated that *TAA1* is essential for a local auxin production in elongating hypocotyl and growing root,[9,10] biological action of TAA in leaves remains unknown. Furthermore, *Os-GI* affected the gene expression of *OsABA8ox1*, *OsYUC6*, *OsLOX2;2*, *OsACS5*, *OsACO3* and *OsIPT4* genes (**Fig. 1**).

Considering the fact in which equal or much higher expression of their palalogous genes in both wild-type and *osgi-1* plants suggests minor effects by *Os-GI* for these steps in each phytohormone biosynthesis, we focused on expression of *OsGA2ox* genes. GA 2-oxidase encoded by *OsGA2ox* genes can deactivate bioactive GAs or its precursors irreversibly to reduce bioactive GA levels.[11,12] Both quantitative analyses of endogenous GA levels and gene expression analyses of GA biosynthetic genes have indicated circadian regulation of GA biosynthesis in other plant species.[13-16] In RiceXPro, a public database of rice gene expression profiling,[17] spatio-temporal gene expression patterns of *OsGA20ox*, *OsGA3ox2* and *OsGA2ox* genes (especially *OsGA20ox2* and *OsGA2ox6*), revealed that leaf organs are one of the major expression sites during rice life cycle. The expression patterns of *OsGA2ox2*, *OsGA2ox3*, *OsGA2ox5* and *OsGA2ox6* in our data generally showed diurnal oscillation that become high during daytime in wild-type leaves (blue lines in **Fig. 2**). Interestingly, this expression pattern is highly similar to the diurnal fluctuation pattern of endogenous GA_8, an inactive GA, in sorghum, another monocotyledonous plant.[13] In contrast, in *osgi-1*, the diurnal expression of these *OsGA2ox* genes were consistently elevated than those of wild-type in all time points tested (red lines in **Fig. 2**). The results suggest that *Os-GI* represses major *OsGA2ox* genes in order to maintain a proper bioactive GA level. Consistently with their elevated gene expression, *osgi-1* plants showed a semi-dwarf phenotype (**Fig. 3A**). We also revealed that the semi-dwarfism in *osgi-1* plants is mainly derived from reduced length of internode and leaf sheath, respectively (**Fig. 3B and C**), corresponding to organs that GA induces elongation to control the rice plant stature.[11] On the other hand, panicle length in *osgi-1* slightly longer than that of wild-type (**Fig. 3D**), suggesting

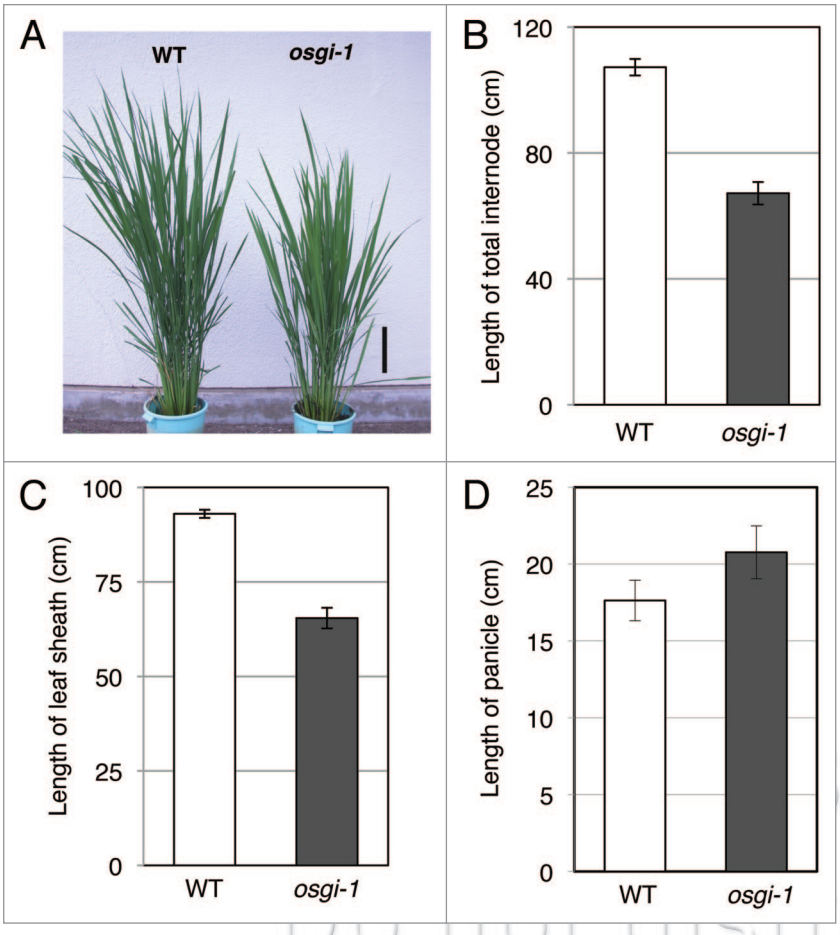

Figure 3. Semi-dwarf phenotype of *osgi-1* plant. (A) Photograph of 90-day-old WT and *osgi-1* plants grown under natural field conditions. Bar indicates 20 cm. (B) Total internode length of WT and *osgi-1* plants. (C) Leaf sheath length at same developmental stage. (D) Panicle length. Bars are expressed as the mean ± SE (n = 9 in B and D, n = 3 in C).

that elevated expression of *GA2ox* in leaves does not affect the development of reproductive organ.[8]

In Arabidopsis, circadian clock regulates rhythmic hypocotyl elongation through transcriptional regulation of the GA receptor genes.[18] However, there were no obvious differences of gene expression of rice GA receptor, *GID1*, and the DELLA protein, *SLR1* (**Fig. 2**).[19] Although the expression level of *OsGA2ox9* was also high in both wild-type and *osgi-1* plants (**Fig. 1**), we could exclude this due to a weak GA deactivation activity of OsGA2ox9.[12]

In ABA actions, there is a consistency of higher *OsSDR* expression with the accumulation of more sucrose and starch in *osgi-1* (**Fig. 1**),[2] since Arabidopsis *ABA2* gene encoding a SDR is induced by sugar treatment and ABA can promote transcription of starch biosynthetic gene in sugar storage processes.[20,21] In addition, *osgi-1* might have more opened guard cells than the wild-type,[2] possibly due to changes in ABA actions. In JA action, whereas JA-Ile, not JA-Me, is the biologically active compound,[22,23] the effect of reduction of *JMT* expression on JA-Ile production remains unknown. Some secondary metabolites accumulated in *osgi-1* are known to be phytoalexin-related flavonoids and stress-inducible,[2,24] suggesting an elevated active JA synthesis in *osgi-1* plants.

In addition to the reduced body size during vegetative growth and longer size of panicle (**Fig. 3**), *osgi-1* plants have an increased numbers of both panicle and spikelet, and with some reduced fertility under stressed conditions.[2] Although quantitative measurements of phytohormones are necessary to confirm the changes of genes, the differences of gene expression reported in this study give us some hints to understand how *Os-GI* modulates growth and development of rice.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

This work was supported by two grants from the Ministry of Agriculture, Forestry and Fisheries of Japan (Genomics for Agricultural Innovation GPN0001 and RTR0004 to T.I.).

Note

Supplemental material can be found at:
www.landesbioscience.com/journals/psb/article/18207/

References

1. Harmer SL. The circadian system in higher plants. Annu Rev Plant Biol 2009; 60:357-77; PMID:19575587; DOI:10.1146/annurev.arplant.043008.092054.
2. Izawa T, Mihara M, Suzuki Y, Gupta M, Itoh H, Nagano AJ, et al. *Os-GIGANTEA* confers robust diurnal rhythms on the global transcriptome of rice in the field. Plant Cell 2011; 23:1741-55; PMID:21571948; DOI:10.1105/tpc.111.083238.
3. Fowler S, Lee K, Onouchi H, Samach A, Richardson K, Morris B, et al. *GIGANTEA*: A circadian clock-controlled gene that regulates photoperiodic flowering in Arabidopsis and encodes a protein with several possible membrane-spanning domain. EMBO J 1999; 18:4679-88; PMID:10469647; DOI:10.1093/emboj/18.17.4679.
4. Park DH, Somers DE, Kim YS, Choy YH, Lim HK, Soh MS, et al. Control of circadian rhythms and photoperiodic flowering by the Arabidopsis *GIGANTEA* gene. Science 1999; 285:1579-82; PMID:10477524; DOI:10.1126/science.285.5433.1579.
5. Itoh H, Nonoue Y, Yano M, Izawa T. A pair of floral regulators sets critical day length for *Hd3a* expression in rice. Nat Genet 2010; 42:635-8; PMID:20543848; DOI:10.1038/ng.606.
6. Thines B, Harmon FG. Four easy pieces: mechanisms underlying circadian regulation of growth and development. Curr Opi Plant Biol 2011; 14:31-7; DOI:10.1016/j.pbi.2010.09.009.
7. Workman C, Jensen LJ, Jarmer H, Berka R, Gautier L, Nielser HB, et al. A new non-linear normalization method for reducing variability in DNA microarray experiments. Genome Biol 2002; 3:48.
8. Hirano K, Aya K, Hobo T, Sakakibara H, Kojima M, Shim RA, et al. Comprehensive transcriptome analysis of phytohormone biosynthesis and signaling genes in microspore/pollen and tapetum of rice. Plant Cell Physiol 2008; 49:1425-50; PMID:18718932; DOI:10.1093/pcp/pcn123.
9. Stepanova AN, Robertson-Hoyt J, Yun J, Benavente LM, Xie DY, Dolezal K, et al. TAA1-mediated auxin biosynthesis is essential for hormone crosstalk and plant development. Cell 2008; 133:177-91; PMID:18394997; DOI:10.1016/j.cell.2008.01.047.
10. Tao Y, Ferrer JL, Ljung K, Pojer F, Hong F, Long JA, et al. Rapid synthesis of auxin via a new tryptophan-dependent pathway is required for shade avoidance in plants. Cell 2008; 133:164-76; PMID:18394996; DOI:10.1016/j.cell.2008.01.049.

11. Sakamoto T, Miura K, Itoh H, Tatsumi T, Ueguchi-Tanaka M, Ishiyama K, et al. An overview of gibberellin metabolism enzyme genes and their related mutants in rice. Plant Physiol 2004; 134:1642-53; PMID:15075394; DOI:10.1104/pp.103.033696.
12. Lo SF, Yang SY, Chen KT, Hsing YI, Zeevaart JAD, Chen LJ, et al. A novel class of gibberellin 2-oxidase control semidwarfism, tillering and root development in rice. Plant Cell 2008; 20:2603-18; PMID:18952778; DOI:10.1105/tpc.108.060913.
13. Foster KR, Morgan PW. Genetic regulation of development in *Sorghum bicolor*. IX. The ma_3^R allele disrupts diurnal control of gibberellin biosynthesis. Plant Physiol 1995; 108:337-43; PMID:12228478; DOI:10.1104/pp.108.1.337.
14. Hisamatsu T, King RW, Helliwell CA, Koshioka M. The involvement of gibberellin 20-oxidase genes in phytochrome-regulated petiole elongation of Arabidopsis. Plant Physiol 2005; 138:1106-16; PMID:15923331; DOI:10.1104/pp.104.059055.
15. Carrera E, Jackson SD, Prat S. Feedback control and diurnal regulation of gibberellin 20-oxidase transcript levels in potato. Plant Physiol 1999; 119:765-73; PMID:9952473; DOI:10.1104/pp.119.2.765.
16. Zhao X, Yu X, Foo E, Symons GM, Lopez J, Bendehakkalu KT, et al. A study of gibberellin homeostasis and cryptochrome-mediated blue light inhibition of hypocotyl elongation. Plant Physiol 2007; 145:106-18; PMID:17644628; DOI:10.1104/pp.107.099838.
17. Sato Y, Antonio BA, Namiki N, Takehisa H, Minami H, Kamatsuki K, et al. RiceXPro: a platform for monitoring gene expression in japonica rice grown under natural field conditions. Nucleic Acids Res 2010; 39:1141-8; PMID:21045061; DOI:10.1093/nar/gkq1085.
18. Arana MV, la Rosa NM, Maloof JN, Blázquez MA, Alabadi D. Circadian oscillation of gibberellin signaling in Arabidopsis. Proc Natl Acad Sci 2011; 108:9292-397; PMID:21576475; DOI:10.1073/pnas.1101050108.
19. Ueguchi-Tanaka M, Nakajima M, Ashikari M, Matsuoka M. Gibberellin receptor and its role in gibberellin signaling in plants. Annu Rev Plant Biol 2007; 58:183-98; PMID:17472566; DOI:10.1146/annurev.arplant.58.032806.103830.
20. Seo M, Koshiba T. Complex regulation of ABA biosynthesis in plants. Trends Plant Sci 2002; 7:41-8; PMID:11804826; DOI:10.1016/S1360-1385(01)02187-2.
21. Rook F, Corke F, Card R, Munz G, Smith C, Bevan MW. Impaired sucrose-induction mutants reveal the modulation of sugar-induced starch biosynthetic gene expression by abscisic acid signaling. Plant J 2001; 26:421-33; PMID:11439129; DOI:10.1046/j.1365-313X.2001.2641043.x.
22. Chini A, Fonseca S, Fernandez G, Adie B, Chico JM, Lorenzo O, et al. The JAZ family of repressor is the missing link in jasmonate signaling. Nature 2008; 448:666-71; PMID:17637675; DOI:10.1038/nature06006.
23. Thies B, Katsir L, Melotto M, Niu Y, Mandaokar A, Lie G, et al. JAZ respressor proteins are targets of the SCF(COI1) complex during jasmonate signaling. Nature 2007; 448:661-5; PMID:17637677; DOI:10.1038/nature05960.
24. Grace SC, Logan BA. Energy dissipation and radical scavenging by the plant phenylpropanoid pathway. Philos Trans R Soc Lond B Biol Sci 2000; 355:1499-510; PMID:11128003; DOI:10.1098/rstb.2000.0710.
25. Mar JC, Wells CA, Quackenbush J. Defining an informativeness metric for clustalling gene expression data. Bioinformatics 2011; 15:1094-100; PMID:21330289; DOI:10.1093/bioinformatics/btr074.