Os-GIGANTEA Confers Robust Diurnal Rhythms on the Global Transcriptome of Rice in the Field

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The circadian clock controls physiological traits such as flowering time, photosynthesis, and growth in plants under laboratory conditions. Under natural field conditions, however, it is known about the significance of the circadian clock in plants. By time-course transcriptome analyses of rice (Oryza sativa) leaves, using a newly isolated rice circadian clock–related mutant carrying a null mutation in Os-GIGANTEA (Os-GI), we show here that Os-GI controlled 75% (false discovery rate = 0.05) of genes among 27,201 genes tested and was required for strong amplitudes and fine-tuning of the diurnal rhythm phases of global gene expression in the field. However, transcripts involved in primary metabolism were not greatly affected by osgi. Time-course metabolome analyses of leaves revealed no trends of change in primary metabolites in osgi plants, and net photosynthetic rates and grain yields were not affected. By contrast, some transcripts and metabolites in the phenylpropanoid metabolite pathway were consistently affected. Thus, net primary assimilation of rice was still robust in the face of such osgi mutation-related circadian clock defects in the field, unlike the case with defects caused by Arabidopsis thaliana toc1 and ztl mutations in the laboratory.

INTRODUCTION

Circadian clocks produce an endogenous rhythm of ~24 h under constant environmental conditions. Recent studies based on molecular genetics using Arabidopsis thaliana as a model plant have revealed that circadian clocks control various physiological traits such as flowering time, plant growth, leaf movement, and guard cell opening in the laboratory (McClung, 2008; Harmer, 2009). For instance, Arabidopsis circadian clocks confer phase-dependent expression of the key flowering regulator CONSTANS (CO) (Putterill et al., 1995; Imaizumi and Kay, 2006). The coincidence of CO gene expression with acute light signals in the evening under long-day (LD) conditions triggers the expression of the floral regulator FT (Corbesier et al., 2007), initiating the floral transition. A recent study of flowering time mutants of Arabidopsis in field environments demonstrated that CO and the circadian clock control flowering time in the field in this species (Wilczek et al., 2009). However, it is largely unknown how plant circadian clocks contribute to the control of various other physiological traits under natural field conditions, in which the environment can differ dramatically from that in the laboratory. An extensive survey of circadian clock–related traits among Arabidopsis accessions collected from various climatic regions revealed broad ranges of natural variations in these traits (Michael et al., 2003), suggesting that natural variation in the Arabidopsis circadian clock may contribute to the adaptation of Arabidopsis to local growing areas (Michael et al., 2003). Although natural variation in flowering time genes involved in vernalization-responsive flowering pathways, such as FLC and FRI, is associated with variations in the local distribution of Arabidopsis accessions, no such associations have been found with natural variation in the genes involved in photoperiodic flowering pathways, such as CO (Shindo et al., 2005, 2006; Izawa, 2007). Therefore, natural variation in circadian clock traits found among Arabidopsis accessions (Michael et al., 2003) may be associated with traits other than flowering time phenotypes. Indeed, circadian clocks have long been believed to influence aspects of primary assimilation, such as photosynthesis, in plants (Harmer, 2009). For instance, it is well known that CAB genes encoding chlorophyll binding a/b proteins are regulated by...
circadian clocks in many plant species under constant environmental conditions (Millar and Kay, 1991; Millar et al., 1995; Sugiyama et al., 2001). In addition, under laboratory conditions, using circadian clock mutants of *Arabidopsis* and cyanobacteria with distinct free-running rhythms, circadian clocks have been shown to influence photosynthesis, growth, and fitness in adapting to artificial rhythms fitted to the free-running rhythms (Ouyang et al., 1998; Green et al., 2002; Dodd et al., 2005). Therefore, plant circadian clocks can influence aspects of primary assimilation under laboratory conditions, although more studies are needed to determine conclusively the biological significance of plant circadian clocks under natural field conditions.

In this work, we first identified a rice (*Oryza sativa*) circadian clock–related mutant by a suppressor mutant screening of late-flowering mutants with a phytochrome deficiency (Izawa et al., 2000) and found that the phenotype was caused by mutations in *GIGANTEA* (Os-GI) (the sole rice ortholog of *Arabidopsis* GI) (Fowler et al., 1999; Park et al., 1999; Hayama et al., 2002). GI is a flowering time gene that is strongly involved in the *Arabidopsis* circadian clock system (Fowler et al., 1999; Park et al., 1999; Locke et al., 2005, 2006; Harmer, 2009). For instance, gi mutants flower very late under LD conditions, because GI is required for phase-dependent CO transcription through the interaction with FKF1, which leads to Cycling DNA binding with one finger (CDF) degradation and consequent derepression of CO (Sawa et al., 2007; Fornara et al., 1999; Hayama et al., 2002).

Because our osgi mutants did not show extreme flowering time phenotypes in the field, we next performed an extensive time-course transcriptome analysis (>27,000 genes tested) and a widely targeted time-course metabolome analysis (>700 assigned chemicals tested) of rice leaves growing in the field (for the methodology of the widely targeted time-course metabolome analysis, see Sawada et al., 2009). We examined the physiological properties of photosynthesis-related traits and yield-related traits in the field to elucidate the genetic roles of Os-GI in the rice circadian clock under natural field conditions. The results demonstrated that the GI-dependent circadian clock confers orchestrated and finely tuned diurnal rhythms on the global transcriptome under natural day–night cycles in rice. Surprisingly, we also demonstrated that, under field conditions, aspects of the primary carbon assimilation of rice, including net photosynthesis, were robust despite the defect in circadian control of the transcriptome due to the osgi mutation, unlike the case under laboratory conditions with defects in *Arabidopsis* circadian clocks caused by *toc1* and *ztl* mutations.

**RESULTS**

**Identification of a Circadian Clock–Related Mutant in Rice**

To elucidate the role of the circadian clock in flowering time control in rice, a short-day (SD) plant, we first screened suppressor mutants of an early-flowering rice mutant that is insensitive to photoperiod, termed photosensitivity5 (se5) (Izawa et al., 2000). The se5 mutant is phytochrome deficient and derepresses the expression of *Heading date 3a* (Hd3a), a rice ortholog of FT (Izawa et al., 2002; Kojima et al., 2002; Tamaki et al., 2007). We hoped to obtain some circadian clock–related mutants because we had previously found that the se5 mutant exhibits fairly normal circadian clock behavior (Izawa et al., 2002). The suppressor mutants that we obtained exhibited late-flowering phenotypes under both SD (10 h light/14 h dark) and LD (14.5 h light/9.5 h dark) conditions (flowering slightly earlier under LD than

![Figure 1](image-url)
under SD) (Figure 1A) and repressed *Hd3a* gene expression, as opposed to the derepressed *Hd3a* expression in se5 mutants (see Supplemental Figure 1 online). Because *Gl* (Fowler et al., 1999; Park et al., 1999) and CO (Putterill et al., 1995) lie upstream of *FT* in Arabidopsis, we examined the expression of *Heading date1* (Hd1) (Yano et al., 2000) (a CO ortholog in rice) and Os-Gl (Hayama et al., 2002). In the suppressor mutants, *Hd1* expression was greater during the day and less at night than in the se5 mutants, and it had a flatter diurnal gene expression. Gl expression was under the limit of detection by RT-PCR in some of these suppressor mutants (see Supplemental Figure 1 online).

We then surveyed the genome sequences of Os-Gl in the suppressor mutants and found one insertion mutation in three mutant lines (Mutant1 [M1], M2, and M3) and one deletion mutation in two mutant lines (M4 and M5) (see Supplemental Figure 2 online). Complementation testing (see Supplemental Figure 3 online) indicated that these mutations caused the suppressor phenotypes. We termed the mutant allele with the insertion *osgi-1* and that with the deletion *osgi-2*. The expression data indicated that *osgi-1* was a null allele of Os-Gl. These suppressor mutations dramatically affected the circadian clock–related genes *PRR1* (Murakami et al., 2003) and *LHY* (Izawa et al., 2002) under both SD and LD conditions (see Supplemental Figure 4 online). The *PRR1* gene in the suppressor mutants was expressed \( \sim 10 \) times more strongly than in the se5 mutant and the wild type at all time points. The *LHY* gene in the suppressor mutants was expressed 100 times less strongly than in the se5 mutant and the wild type at 02:00 and 04:00, whereas the expression levels were similar during the daytime. These findings indicated that the circadian clocks in the suppressor mutants were severely impaired.

We then crossed the M2 mutant line (se5 *osgi-1*) with the *hd1*-deficient near-isogenic line NIL(*hd1*-Kas) (Yano et al., 2000), and we examined flowering time in the progeny (Figure 1B). The single *osgi-1* mutation extended the time to flowering only under SD conditions. Under LD conditions the single *hd1* mutation induced early flowering, but *osgi-1* plants exhibited no phenotypic change, strongly suggesting that the roles of *Gl* in floral regulation are distinct from those of *Hd1* in rice. The flowering time phenotypes of the *hd1 osgi-1* double mutants were intermediate between those of the *hd1* and *osgi-1* single mutants.

**Flowering Time Phenotypes of osgi Mutants in the Field**

In our studies, *osgi* mutants exhibited late flowering under SD conditions but no change under LD conditions (Figure 1B). We then asked whether Os-Gl contributes to rice flowering time in the field, since we knew that, in typical rice cultivation in temperate areas of Asia, floral transition occurs under natural LD conditions (Izawa, 2007). We reared \( \sim 1000 \) F2 progeny of a cross between *se5 osgi-1* and Kasalath (an *aus* rice subgroup cultivar with an *hd1*-deficient allele) were grown. (A) Os-Gl. All plants were *Hd1/hd1* and *Se5/se5*. Red, *osgi/osgi* (N8 type); white, *OsGl/osgi* (hetero); blue, *OsGi/Osgi* (Kas type). (B) *Hd1*. All plants were *OsGi/osgi* and *Se5/se5*. Red, *Hd1/Hd1* (N8 type); white, *Hd1/hd1* (hetero); blue, *Hd1/Hd1* (Kas type). (C) *Se5*. All plants were *Hd1/hd1* and *Osgi/osgi*. Red, *se5/se5* (N8 type); white, *Se5/se5* (hetero); blue, *Se5/Se5* (Kas type).
transmitting dates (achieved by staggering transplanting dates at 1-week intervals), and we performed a series of microarray analyses with an Agilent 44K rice microarray chip. On the sampling date, environmental conditions, such as sunlight and temperature, were also monitored to determine whether the ambient environmental conditions on the sampling date were in the physiological range for rice growth (see Supplemental Table 1 and Supplemental Figure 7 online). The photoperiods were estimated from the global solar radiation on each sampling date (see Supplemental Table 1 online) and are plotted as day–night bars in Supplemental Figures 8 to 10 online. In summary, 104 arrays were used, with two replicates at each of 13 time points and four developmental stages (2 × 13 × 4 = 104). On each array, the wild-type sample was labeled with Cy3 and the osgi sample was labeled with Cy5. (See Methods and Supplemental Data Set 2 online for assessment of the dye effect when using the rice 44K Agilent microarray.) We did not focus on the differences among developmental stages and instead averaged (or considered together) all eight microarray data sets (two replicates × four developmental stages) per time point to mask the developmental effects. The major conclusions (Figure 3) drawn from the rice diurnal transcriptome data in the field were obtained with these averaged data. For statistical comparison, an unpaired t test assuming equal variances was performed between the eight sets of processed data for the wild type and osgi per time point to judge the effect of Os-GI on the expression of each tested gene at each time point (see Supplemental Figure 11 and Supplemental Tables 2 and 3 online; also see below). We therefore considered that we had sufficient biological replicates to draw valid conclusions. We finally obtained 206 microarray data sets (Cy3-labeled for the wild type and Cy5-labeled for osgi-1). (We discarded one data set because of high variance.) All data are publicly available in the SALAD on ARRAYs viewer (Mihara et al., 2010) of our database (http://saldn.affrc.go.jp/salad/) and also have been deposited to the Gene Expression Omnibus under accession number GSE18685.

With these data, we first checked circadian clock–related gene expression. (Note that the graphs are presented in log2 scales [see Supplemental Figures 8 to 10 online].) The dynamic ranges of data processed for Os-GI, -LHY, and -PRR1 in the wild type covered two, three, and two orders of magnitude, respectively (see Supplemental Figure 8 online) and were basically consistent with the quantitative RT-PCR results in se5 osgi-1 plants (see Supplemental Figures 1 and 4 online). At all time points, GI expression in osgi-1 plants was around one order of magnitude less than that in wild-type plants. This reduced level of GI gene expression could have been due to nonsense-mediated decay of mRNA caused by the insertional mutagenesis (see Supplemental Figure 2 online) because the order of amplitude and the rhythm pattern of GI expression did not differ between the wild-type and osgi-1 plants; the only difference was in the level of GI expression. Therefore, it is likely that the rhythmic transcriptional regulation of Os-GI works independently of GI function. Os-LHY expression was substantially reduced at all time points in osgi-1 plants (see Supplemental Figure 8 online); at some points (e.g., midnight), there was more than one order of magnitude difference from the value in wild-type plants, but a clear rhythm of Os-LHY expression remained in the osgi-1 plants. By contrast, Os-PRR1 was substantially upregulated in osgi-1 plants at many points (except at 15:00, 17:00, and 19:00), but its expression exhibited a relatively flat pattern with a reduced rhythm (see Supplemental Figure 8 online). These results indicated that the circadian clock was severely impaired in osgi-1 plants, even under the natural day–night conditions in the field. In rice, GI negatively regulates PRR1, although mathematical modeling in Arabidopsis suggests that At-GI activates the ortholog of Os-PRR1, At-TOC1 (Strayer et al., 2000), and it has been experimentally shown that this activation might occur through post-translational regulation of TOC1 protein by GI and ZTL (Locke et al., 2005; Kim et al., 2007; Martin-Troyon et al., 2007; Pokhilko et al., 2010). The role of Os-GI in the rice circadian clock clearly differs from that of At-GI in Arabidopsis, since the effects of a gi single mutation on TOC1 transcription in Arabidopsis are not detectable and those on other circadian clock–related genes are subtle and unclear under diurnal conditions (Kim et al., 2007; Martin-Troyon et al., 2007). As with Os-PRR1, clearly damped rhythms of Os-PRR95 and Os-PRR99 diurnal expression were also observed in osgi-1 plants (Murakami et al., 2003), although the difference was greater from evening to dawn than that in Os-PRR1 (see Supplemental Figure 8 online). By contrast, there were no effects on rhythmic expression patterns of Os-PRR73 or Os-PRR37 (Murakami et al., 2003) (see Supplemental Figure 8 online), suggesting that Os-GI does not regulate these PRR genes (i.e., PRR73 and PRR37) in rice. If we assume that there is some evolutionary conservation between rice circadian clock–related genes and those associated with Arabidopsis circadian clocks, where the circadian clock consists of interlocked subloops, it is also possible that Os-GI is a member of a subloop in rice circadian clocks. If this is the case, because the amplitudes and expression patterns of the rhythmic transcription patterns of Os-LHY and Os-GI were not affected severely by the osgi-1 mutation, both Os-LHY and Os-GI may themselves function as interlocks. By contrast, Os-PRR1, -PRR59, and -PRR95 require Os-GI to maintain their strong rhythms and may be members of a subloop in which GI is a central member, whereas PRR73 and PRR37 may consist of other subloops independent of GI.

Consistently, expression of some other circadian clock–related genes, such as LHY-related genes (Os04g0583900 and Os02g0685200), LUX (Os01g0971800), and ELF3like
Os01g0566100 was affected by osgi-1 (see Supplemental Figure 9 online). Like PRR1, FKF1 (Os11g0547000) was upregulated in osgi plants, with a damped rhythm, but LKP2 (Os02g0150800) and ZTL (Os06g0694000) were not affected (see Supplemental Figure 9 online). Therefore, Os-FKF1 may function as a downstream gene in the Os-GI–dependent clock. Alternatively, it could be a member of the Os-GI–dependent subloop itself.

The major flowering time gene Hd1 was affected in the evening and at night by the single osgi-1 mutation; this was consistent with the RT-PCR data for se5 osgi-1 (see Supplemental Figures 1 and 10 online). Some CO-like and COL9-like genes were dramatically affected by osgi-1 (see Supplemental Figure 10 online), suggesting that the rhythmic expression of several output genes is regulated critically by Os-GI–dependent circadian clocks in rice.

To elucidate how these changes in circadian clock–related genes affect global gene expression in osgi mutants in the field, we next checked the effect of the osgi mutation on the entire rice transcriptome in field-grown plants. Among the 27,201 nonredundant tested genes, the expression of 57% (at false discovery rate [FDR] = 0.01) or 75% (FDR = 0.05) was significantly affected by osgi-1 under natural diurnal conditions (see Supplemental Figure 11 and Supplemental Tables 2 and 3 online). Here, significance was examined by t test among the eight log2-calibrated data sets from the processed data of the Agilent rice 44K array for each time point (see Supplemental Figure 11 online).

Figure 3. Field Transcriptome Analysis of osgi Mutant.

Hundreds of rice plants were grown in a paddy field (2008; Tsukuba, Japan). Leaf samples were harvested from several plants at 2-h intervals from 07:00 on August 12th to 07:00 on August 13th.

(A) to (C) Heat maps of pairwise Pearson’s coefficients for averaged processed data of 27,201 genes (Cy3- or Cy5-labeled). From high to low values, colors change in the order of green–orange–red. From left to right (and top to bottom), the orders of bins were from developmental stages 1 to 4 (each with a 1-week difference in transplanting dates) at each indicated time. Time point order was from 7:00 August 12th to 7:00 August 13th. WT, wild type.

(A) For 103 microarray data from the wild type (or Norin 8): 13 time points × 2 leaves × 4 stages.

(B) For 103 microarray data from osgi: 13 time points × 2 leaves × 4 stages. (For one sample taken at 19:00, stage 4 was removed because of abnormal variance.)

(C) Relationship between the wild type and osgi. Phases were shifted.

(D) and (E) Averaged coefficients of eight samples per time point were plotted, from data of (A) from the wild type (D) and from data of (B) from osgi (E). Lines are labeled with the corresponding time points.
observed in osgi-1 plants (Figures 3B and 3E; see Supplemental Figure 12 online). The changes in the coefficients of correlation in a day were clearly greater in wild-type plants than in the osgi mutants, indicating a more drastic phase dependency and stronger amplitudes of the transcriptomes in wild-type plants than osgi-1 plants (Figures 3D and 3E).

We next used cosine curve regression models to estimate the peak phase corresponding to a 24-h period for all tested genes using nonlinear least-squares methods (see Supplemental Figures 13 and 14 online). For this cosine curve fitting, for which we used data averaged over eight microarray data sets, we adopted an original score, termed a fitting score, to select genes that were diurnally expressed in rice (see details of fitting scores in Methods). Our aim was to select genes with diurnal gene expression that was as global as possible; from the transcriptome data obtained from rice leaves, we selected >6000 genes that were diurnally expressed.

The peak phases of expression of these selected diurnally expressed genes were severely affected in the osgi-1 mutants; in osgi plants, we found two major gene groups with high expression amplitudes and either a morning or night peak, but in wild-type plants, the peak phases with high amplitudes were distributed more evenly (see Supplemental Figure 15 online). By contrast, gene groups with low amplitudes were evenly distributed in the osgi mutants, and no group of low-amplitude genes with a midday peak was clearly observed in the wild-type plants (see Supplemental Figure 15 online). In addition, some phases of each type of gene expression were delayed or advanced in the osgi plants compared with those in the wild type at all time points (see Supplemental Figures 16 and 17 online). For the majority of genes, the amplitudes of expression (corresponding to “b” in the regression models; see also Supplemental Figure 13 online) were reduced in the osgi plants (see Supplemental Figure 18 online). Fitting to the cosine curves also differed between wild-type and osgi plants; some genes showed better fitting in wild-type plants, whereas others fitted better in the osgi mutants. Basal expression (corresponding to “a”) did not change greatly (see Supplemental Figure 18 online). These findings indicated a close relationship between Os-GI and the transcriptome profile (i.e., the phase setting and amplitude of the transcriptome). These data clearly demonstrated that Os-GI confers fine phase-setting and strong amplitudes of orchestrated global diurnal rhythms on the transcriptomes of rice leaves in the field (Figure 3; see Supplemental Figures 12 and 15 to 18 online).

Physiological Phenotypes of osgi Mutants in the Field

We next examined the photosynthetic properties and diurnal changes in photosynthetic pools under full sun (Figure 4; see Supplemental Table 4 online) because circadian clocks are believed to maintain photosynthetic performance and the resulting primary production. In Arabidopsis, artificial environments matched to a free-running period enhance photosynthesis in long and short circadian period mutants (Dodd et al., 2005). Our results clearly demonstrated that the contents of Suc and starch in the leaves were significantly increased at most time points in osgi-1 mutants (Figures 4F and 4G). Furthermore, leaf nitrogen content and light-saturated photosynthesis at different CO₂ partial pressures were not affected in osgi-1 plants (Figures 4A and 4D). These findings implied that loss of proper phase setting and changes in the amplitude of transcriptome expression resulted in some increase in primary production in the leaves of osgi mutants of rice, although the physiological and biochemical functions of Os-GI protein are still largely unknown. Increased starch content is also reported in gi mutants in Arabidopsis (Eimert et al., 1995). A similar, but more extensive, analysis of metabolite profiling has been reported in Arabidopsis gi mutants (called starch excess3 mutants by the authors; Messerli et al., 2007). Chlorophyll content was significantly reduced in osgi plants (Figure 4E), but stomatal conductance was significantly increased, resulting in significantly increased intercellular CO₂ partial pressures (Figures 4B and 4C) (Seneweera et al., 1995). However, these changes did not affect the photosynthetic rate (Figure 4A).

We then surveyed our microarray data for photosynthesis-related pathways and primary metabolic pathways (Figure 5; see Supplemental Figures 19 to 28 online). In the pathways regulating sink size, several starch synthesis–related genes were clearly upregulated in osgi plants (Figure 5; see Supplemental Figure 19 online). Consistently, the main starch degradation genes were downregulated, especially in the morning (Figure 5; see Supplemental Figure 20 online). These results indicate clearly that Os-GI can affect the main starch metabolic genes to control starch accumulation, although it is still possible that Os-GI can also affect enzymatic activity in starch metabolic pathways. In the other primary metabolism pathways and photosynthetic and mitochondrial electron transfer pathways, no critical changes in gene expression and no trends—even when there were changes in primary metabolite production—were observed (Figure 5; see Supplemental Figures 21 to 28 online). The orchestrated transcriptome diurnal rhythms set by Os-GI are therefore not essential for high levels of photosynthetic efficiency in rice. (Note that the data presented in the graphs in Supplemental Figures 19 to 29 are those in which the ratio of osgi to the wild type [or the wild type to osgi] was >2 and the mRNA level in wild-type plants was higher than the background hybridization level.)

In addition, panicle numbers and spikelet numbers were significantly increased in osgi-1 plants (Figures 4H and 4I), possibly because of outgrowth of axial meristems and increased panicle formation using the increased amounts of photosynthate. However, because osgi grains were significantly lighter than wild-type grains, grain yield was not affected in osgi-1 plants (Figures 4J and 4K). In contrast with the case with primary assimilation, expression of some key genes in the phenylpropanoid pathway (a secondary metabolite pathway controlled by circadian clocks; Harmer, 2009) was significantly upregulated in osgi plants (see Supplemental Figure 29 online). Furthermore, under atypical growing conditions with late transplanting dates in the field, fertility was significantly reduced in osgi plants, indicating some loss of seasonal adaptability (Figures 4L and 4M).

Metabolome Analysis of osgi Mutants in the Field

To further elucidate the physiological roles of Os-GI, we performed a widely targeted metabolome analysis (see Methods in Sawada
et al., 2009) of rice leaves growing in the field. Among ~700 chemicals tested, we found significant changes in the production of 73 metabolites in osgi plants relative to the wild type (N8). (See our summary database of this metabolome analysis in Supplemental Data Sets 3 and 4 online.) In Supplemental Data Set 4 online, for the 73 metabolites we plotted the diurnal time-course changes in mass peak areas, as well as mean values, difference plots, and density plots. In these metabolome results, among metabolites in the tricarboxylic acid cycle, the production of malate, citrate, and 2-oxoglutarate was significantly decreased in osgi-1 plants, whereas aconitate and isocitrate production was significantly increased (Figure 6). Levels of succinate and fumarate were not significantly affected in osgi-1 leaves (Figure 6). In addition, we observed an increase in flavonoid production and a clear reduction in Phe production (see Supplemental Figure 30 online). The reduction of fertility in osgi plants under atypical growing conditions (Figures 4L and 4M) was consistent with this accumulation of flavonoids in osgi leaves (see Supplemental Figure 30 online) because some of these flavonoids are known to be stress inducible (Grace and Logan, 2000).

**DISCUSSION**

**OsGI Confers Fine Phase Setting and a Strong Orchestrated Rhythm on the Global Transcriptome in the Field**

In this work, we focused on the physiological roles of plant circadian clocks under natural field conditions. The traits of circadian clocks, including free-running rhythms, have long been examined under constant environmental conditions, ever since cab:luc screening of circadian clock mutants of Arabidopsis was successfully performed (Millar et al., 1995; McClung, 2008; Harmer, 2009). Assaying under constant light (LL) or constant dark (DD) is done mainly to be able to more clearly quantify the endogenous period of the traits and to elucidate the self-oscillation mechanisms, where free-running assays are simply to obtain better period estimates. However, the phenotypes observed

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**Figure 4.** Photosynthesis, Primary Metabolism, and Yield-Related Traits in osgi.

(A) CO₂ assimilation rates (A) at different [CO₂] partial pressures. Measurements were made at an ambient CO₂ pressure (pCa) of 39 Pa and intercellular CO₂ pressures (pCi) of 20 and >60 Pa.

(B) Stomatal conductance (gₛ) measured at pCa = 39 Pa.

(C) Intercellular CO₂ partial pressure (pCi) measured at pCa = 39 Pa.

(D) Leaf nitrogen (N) content.

(E) Leaf chlorophyll (Chl) content.

(F) Diurnal change in leaf starch content. WT, wild type.

(G) Diurnal change in leaf Suc content. Each datum is represented as the mean ± SE from 4 to 5 independent experiments in (A) to (G).

(H) Panicle numbers per plant.

(I) Spikelet numbers per plant. Significant difference (asterisk) at P < 0.05.

(J) Averaged grain weights.

(K) Grain yields per plant.

(L) Averaged fertility.

(M) Fertility under atypical growing conditions with late transplanting dates. Numbers in parentheses indicate the transplanting dates. Significant difference is indicated as *P < 0.05 and **P < 0.01.

[See online article for color version of this figure.]
under such constant environmental conditions do not necessarily reflect the biological roles of circadian clocks under natural day–night cycles of sunlight or diurnal artificial conditions in the laboratory. Because circadian clocks are believed to control target genes and, hence, related traits under natural diurnal conditions, we performed transcriptome analyses, targeted metabolome analyses, and physiological analyses of photosynthetic properties using osgi mutants of rice.

In Arabidopsis, GI is thought to be a circadian clock component gene (Fowler et al., 1999; Park et al., 1999; Locke et al., 2005).

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**Figure 5.** Robustness of Gene Expression Related to Primary Assimilation to the osgi Mutation.

Using color gradients as described in the figure, average mRNA levels during the 24 h in the wild type and their ratios between the wild type (WT) and osgi are indicated as left and right boxes with different colors, respectively. From left to right columns, all gene expression related to starch synthesis, starch degradation, TCA cycle, Calvin cycle, Suc metabolism, oxidative reaction of pentose phosphate pathway (pentose phosphate) (in cytosol [Cyt] and chloroplast [Chl]), glycolysis I (Glyco I), and glycolysis II (Glyco II). Photochemical reaction and electron transfer in chloroplast (Chl) and mitochondrion (Mito) are summarized. Detailed data are also shown in Supplemental Figures 19 to 28 online. Less than one-tenth and >10 ratios (osgi/wild type) were not observed among these genes. Numbers in parentheses in a column indicate an enzyme in the pathway corresponding to those in Supplemental Figures 19 to 28 online.
Here, we demonstrated that Os-GI, which is transcriptionally regulated by diurnal light changes in the laboratory (Itoh et al., 2010), is required for strong amplitudes and fine-tuning of the diurnal rhythm phases of global transcriptome expression under natural day–night field conditions (Figure 3; see Supplemental Figures 15 to 18 online). It is generally believed that ambient environmental cues may control the entrainment of circadian clocks. Therefore, this concept, together with our results, suggests that entrained circadian clocks require the function of Os-GI to give rise to strong diurnal rhythms and fine phase-setting of global transcriptome expression. Considering the changes in circadian clock–related gene expression in the osgi mutants, it is possible that Os-GI participates in a subloop of circadian clocks in rice and uses ambient environmental signals to entrain the subloop clocks to produce the strong, finely tuned, and orchestrated rhythms in the transcriptomes of rice (see Supplemental Figures 15 to 17 online). Consistent with this idea, darkness longer than normal night dramatically reduces rhythmic gene expression of Os-GI in the laboratory (Itoh et al., 2010). On the other hand, even in the osgi mutants, there remained two distinct transcriptomal profiles (i.e., day and night; Figure 3), although the phase settings and amplitudes of rhythmic gene expression were severely impaired in these plants. Therefore, in osgi mutants, diurnal changes in environmental cues can cause only all-or-none-type responses in the transcriptome profiles, with no fine phase setting. Taken together, our results indicate that Os-GI confers fine phase setting and a strong orchestrated rhythm on the global transcriptome in the field.

Figure 6. Summary of Changes in Levels of Primary Metabolites in osgi.

The analyses were performed based on the results of a widely targeted metabolome analysis (Sawada et al., 2009). Summarized data from Supplemental Data Sets 3 and 4 online for primary metabolites are presented. Colors of circles represent the fold changes (osgi/wild type) as indicated in the figure. Sizes of circles represent FDRs. FDR was calculated using the P values of a paired t test with 52 values (2 × 13 time points for both the wild type and osgi).

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2006; Kim et al., 2007; Pokhilko et al., 2010). Although light and ambient temperature are known to be zeitgebers in the laboratory, the diurnal changes in environmental cues responsible for entraining circadian clocks have not been identified experimentally under natural field conditions. For example, we do not know which signal (dawn or dusk) has the stronger effect on the entrainment of circadian clocks in the field. Extensive mathematical modeling to elucidate the relationships between various environmental cues and gene expression in our transcriptome data are now under investigation in our laboratory, with the aim of determining which environmental cues are the most effective in entraining circadian clocks or gating circadian clock–controlled genes in rice.
**Associations between Transcriptome and Metabolome Data of osgi**

One of the major actions of the circadian clock in *Arabidopsis* is thought to be the transcriptional regulation of downstream genes. Currently, a negative feedback transcriptional loop between CCA1/LHY and TOC1 (or other PRRs) is believed to be the core clock mechanism in *Arabidopsis* (Strayer et al., 2000; Alabadi et al., 2001). Here, because we obtained both transcriptome and annotated metabolome data from osgi leaves in rice under field conditions, we used the osgi-1 mutation to address the simple question of how changes in circadian clock–controlled gene expression in some metabolic pathways were associated with the levels of related metabolites under field conditions. In Arabidopsis, it is well known that the transcription of genes in the phenylpropanoid pathway is under the control of circadian clocks (Harmer, 2009). As expected from this knowledge, in our transcriptome data, expression of several genes of the phenylpropanoid pathway was significantly affected by the osgi-1 mutation under natural diurnal conditions in the field; the majority of these genes were upregulated (see Supplemental Figure 29 online). Because only the net effects of the osgi-1 mutation on the expression of paralogous genes imply some ability to change the production of related metabolites, we checked the expression of all paralogous gene members in the rice genome (Figure 5; see Supplemental Figures 19 to 29 online). Consistent with the transcriptome data on the phenylpropanoid pathway (see Supplemental Figure 29 online), we observed that the production of several metabolites in the phenylpropanoid pathway was significantly increased (see Supplemental Figure 30 and Supplemental Data Set 4 online). By contrast, the pool of Phe, a major chemical precursor in the phenylpropanoid pathway, was significantly decreased in our metabolome data. We found a similar association between a set of transcriptome data and the related metabolome data on starch metabolism (Figures 4F, 4G, and 6; see Supplemental Figures 19 and 20 online). These examples suggest that this transcriptional regulation primarily influences the amounts of the corresponding products in both the starch and the phenylpropanoid metabolic pathways in rice. Note that the possibility of posttranslational regulation of starch metabolic enzymes by circadian clocks was not excluded by our results. Some of these changes in the starch production pathway were consistent with those in previous reports (Zeeman et al., 2007; Fulton et al., 2008).

Recently, extensive work has been done on starch metabolism and circadian clocks in *Arabidopsis* (Fukushima et al., 2009; Graf et al., 2010). Graf et al. (2010) reported that starch degradation during the night, which is a determinant of growth in *Arabidopsis*, is partly under circadian control. This observation is consistent with our finding that some amylase (starch degradation enzyme) genes of rice were expressed at lower levels in the leaves of osgi mutants than in those of the wild type (see Supplemental Figure 20 online). Fukushima et al. (2009) reported that a triple PRR mutant of *Arabidopsis, d975*, showed considerably increased production of TCA cycle intermediates, whereas a CCA-over-expressing (CCA-ox) mutant showed less change in primary metabolism. Because the *Arabidopsis* circadian clock does not cycle in CCA-ox mutants (Wang and Tobin, 1998), we considered that the reduced effects of the transcripts on primary metabolism in osgi mutants were consistent with the CCA-ox phenotype. Because the d975 mutant shows dramatic changes in the diurnal expression of many genes under diurnal conditions in the laboratory (Nakamichi et al., 2009), the upregulation of TCA cycle member genes suggests that both light signaling and the circadian clock are impaired in d975 mutants.

In contrast with the above examples, transcript changes related to the production of other major primary metabolites revealed no clear associations with the changes in related metabolome data in rice (Figures 5 and 6; see Supplemental Figures 21 to 28 online). For example, production of malate, citrate, and 2-oxoglutarate in the TCA cycle was significantly decreased, whereas ascorbate and isocitrate production was increased (Figure 6). These changes in primary metabolite production in the TCA cycle were not supported by our transcriptome data because the expression of most genes encoding TCA cycle enzymes was not critically changed in the osgi mutants (Figure 5; see Supplemental Figure 21 online). Furthermore, levels of succinate and fumarate in the TCA cycle were not significantly affected in osgi mutants (Figure 6). Considering the relatively normal growth of osgi plants in the field (Figure 4), it is likely that the TCA cycle itself was not impaired in the osgi mutants. The changes in the amounts of these primary metabolites might have been brought about indirectly as a result of changes in their pool sizes through related secondary metabolism. For example, levels of both Phe (a primary metabolism product) and related secondary metabolites were affected together (see Supplemental Figure 30 and Supplemental Data Set 4 online). These data strongly indicate that the expression of the majority of genes related to primary metabolites was robustly controlled by homeostasis at the transcriptional level. Therefore, in considering transcriptional regulation as a primary output of circadian clocks, we concluded that the osgi mutation did not alter primary productivity (Figures 4 to 6), even though in the transcriptome analysis the expression of 57% (FDR = 0.01) or 75% (FDR = 0.05) of genes was significantly affected by the osgi mutation under natural diurnal day–night conditions in the field (see Supplemental Figure 11 online). In addition, circadian clock–dependent modification of the activity of enzymes associated with primary metabolite production would explain the lack of association between transcriptomes and metabolomes, although such modifications are not yet considered as major outputs of plant circadian clocks.

**Robustness of Primary Assimilation in osgi Mutation in the Field**

The finding that the osgi mutation did not greatly affect the flowering time of rice in the field (Figure 2; see Supplemental Figures 5 and 6 online) raised the question of the physiological roles of the circadian clock in rice under natural day–night conditions. For instance, the ambient light intensity to which plants are subjected in the laboratory is generally weak (≈50 to 200 μmol m⁻² s⁻¹). By contrast, in the field on a typical sunny day (for instance, in Tsukuba, Japan), the sunlight intensity to which plants are subjected is often up to 2000 μmol m⁻² s⁻¹. Therefore, we decided to examine osgi mutants under field conditions to elucidate the physiological roles of Os-GI in the plant circadian
clock. Here, we clearly demonstrated that, even under natural day–night cycle conditions, Os-GF1 gave a clear, orchestrated diurnal phase dependency to rice global transcriptomes, producing marked differences in expression at different time points (Figure 3). Unexpectedly, the net photosynthesis rate was not affected by the global defect caused by the osgi mutation (Figures 4A to 4E), indicating the robustness of primary assimilation in the face of defects in the circadian clock. This conclusion was further supported by the field transcriptome data (Figure 5), the widely targeted time-course metabolome analysis (Figure 6), and the examination of yield related traits in the field (Figures 4H and 4I). These results strongly suggest that the genetic diversity of circadian clock–related traits contributes to the local adaptation of rice cultivars through traits other than those related to primary assimilation.

Physiological changes in flowering time control and some secondary metabolic pathways of stress response are likely candidate traits related to such local adaptations. For instance, osgi mutants become late flowering under SD conditions (Figure 1B), suggesting that there would be changes in flowering time if these mutants were grown in tropical areas. In the field, natural cues (daylight, temperature, and photoperiod) often fluctuate, sometimes leading to abiotic and biotic stresses on plants. Indeed, we showed that, under atypical growing conditions with late transplanting dates in the field, fertility was significantly reduced in osgi plants, indicating loss of seasonal adaptability (Figures 4L and 4M). The accumulation of phytoalexin-related flavonoids, such as 3-deoxyanthocyanidins in osgi leaves (see Supplemental Figure 30 online), could be a response to natural stress because some flavonoids are stress inducible (Grace and Logan, 2000).

Overall, as shown here, by using circadian clock functions, rice plants maintain strong amplitudes and fine-tuning of the diurnal rhythm phases of global transcriptomes, thus likely enabling them to better adapt to fluctuating environmental conditions in the field. However, photosynthesis- and growth-related primary assimilation in rice was robust in the face of a severe defect in the diurnally rhythmic transcriptomes under field conditions (Figures 4 and 5). It is likely that plant primary assimilation ability is firmly protected by some unidentified homeostatic feedback system. In Arabidopsis under dim light conditions in the laboratory, the circadian clock plays an important role in maintaining primary assimilation (Ouyang et al., 1998; Green et al., 2002; Dodd et al., 2005; Harmer, 2009). This suggests that plant researchers should be more keen to use ambient environmental conditions to investigate the biological roles of circadian clocks.

METHODS

Suppressor Mutant Screening

About 3000 se5 seeds were irradiated with a conventional dose of γ-rays (Institute of Radiation Breeding) and then germinated to give M1 plants. The M2 seeds obtained were bulk harvested into six groups. In total, 18,000 M2 plants were grown in a paddy field and screened for flowering phenotype. Eight individuals were isolated as suppressor mutants; five were analyzed in this work. M1, M2, and M3 plants were obtained from one group in the M2 population, and M4 and M5 plants were obtained from another group.

Genotyping of Flowering Time Loci

After transplantation of the rice (Oryza sativa) plants, part of a leaf from each of 1000 tested plants was sampled and used for DNA preparation. Fragment analysis using GeneMapper software (Genetic Analyzer ABI3130) was performed for genotyping. The primers strongly linked to target loci were RM5552F (5'-AGCCCAAGGGAGTAATTACA-ACC-3') and RM552R (5'-GGTTCAAGATATGATGCTGAAAGC-3') for Os-GF1; RM6836F (5'-AGCCGTGCAAAATTAGATACCGG-3') and RM6836R (5'-GTGTGCTCAGCCAGTATGTTGTC-3') for Hdt1; and RM3628F (5'-AGCCCTAGACACCCGTACCC-3') and RM3628R (5'-GTGTGCCAGATGAAAATATG-3') for Se5.

PCR fragments were postlabeled for detection.

Transcriptome Analysis

For microarray analysis, rice leaves were sampled in a paddy field every 2 h from 07:00 on August 12, 2008 to 07:00 on August 13, 2008. Ambient conditions on August 12th to the 13th (2008) are shown in Supplemental Figure 7 online. Photoperiods were estimated from the global solar radiation (see Supplemental Table 1 online); the corresponding day–night bars are shown in Supplemental Figures 8 to 10 online. Both wild-type and osgi plants flowered in the first week in September. RNA was prepared with an RNeasy plant mini kit (Qiagen). A rice 44K Agilent microarray chip (Agilent Technologies) was used. RNA quality in all cases was analyzed using Nanodrop (Thermo Fisher Scientific) and Bioanalyzer (Agilent Technologies). RNA was labeled in accordance with the manufacturer’s protocol (the wild type with Cy3 and osgi with Cy5). Cy3- and Cy5-labeled cRNA probes (800 ng) were hybridized to a single chip. All microarray analyses were systematically performed by the same researcher (T. Izawa) and a technical assistant (R. Motoyama) in a series of experiments (four chips for one experiment). Redundancy of distinct probes for the same gene in the chip was considered to summarize the data into the expression of 27,201 distinct genes. If multiple probes existed, the averaged processed data were considered to represent expression of the corresponding gene.

Normalization and Correlation Analysis

For each set of microarray data, to reduce the indeterminant error among microarray experiments, we first normalized the obtained data using the qspline method, which was implemented in R and included in the Bioconductor package (http://www.r-project.org/; Workman et al., 2002). Normalized data were analyzed with Excel (for heat map display) and R software (for correlation coefficient calculation and two-dimensional histogram display).

f Test

The unpaired f test assuming equal variances was used for statistical comparison between the eight sets of processed data for wild-type and osgi plants at a time point. Both FDR = 0.01 and FDR = 0.05 were considered significant (see Supplemental Figure 11 and Supplemental Table 3 online). A total of 27,201 × 13 P values were used for this FDR calibration (Benjamini and Hochberg, 1995). When any significant difference at one time point or more among 13 time points was detected for a tested gene by this f test, we concluded that the gene was regulated by the Os-GF1 gene.

Fitting Methods

Normalized data were transformed to log2 values. We applied three curve-fitting models over microarray data for 13 time points using
nonlinear least squares in R (http://www.r-project.org/) (see also Supplemental Figure 14 online):

Model 1: \[ y = a + b \times \cos \left( \frac{2\pi t}{12} + \vartheta_1 \right) \]

Model 2: \[ y = a + b \times \cos \left( \frac{2\pi t}{12} + \vartheta_1 \right) + c \times \cos \left( \frac{2\pi t}{6} + \vartheta_2 \right) \]

Model 3: \[ y = a + b \times \cos \left( \frac{2\pi t}{12} + \vartheta_1 \right) + c \times \cos \left( \frac{2\pi t}{6} + \vartheta_2 \right) + d \times \cos \left( \frac{2\pi t}{3} + \vartheta_3 \right) \]

where \( y \) represents the microarray data and \( t \) is the time, \( a, b, c, d, \vartheta_1, \vartheta_2, \) and \( \vartheta_3 \) are estimate parameters, and \( \vartheta_1 \) is the phase estimate for the entrained 24-h period.

Model Evaluation

We compared \( \vartheta_1 \) and the peak points of the fitted model curves between Model 1 and the other two models. Theoretically, \( \vartheta_1 \) should be identical among models because these models are based on Fourier transformation formulae. This was confirmed in our cases by nonlinear least squares (see Supplemental Figure 13 online).

Fitting Evaluation

Fitted model curves were evaluated using a fitting score, which was defined as residual standard error divided by amplitude \([\text{i.e.,}\ \text{sort} \left( \text{Dev}/n - 3/\text{sd} \right)]\).

Here, Dev, \( n \), and sd indicate residual sum of squares, total number of time points, and amplitude, respectively. A low fitting index indicates good fit. The basic idea here is very simple. The residual sum of squares (or residual standard error) can be considered as noise in the experiments. Therefore, the ratio of noise against b (amplitude) can be used as a simple score to explain how a value is fitted to a cosine curve. We empirically set 0.6 as a threshold fitting score (see Supplemental Table 4 online). To justify this criterion, we present examples of best fits, borderline fits, and worst fits in Supplemental Figure 14 online.

Dye Effect of Cy5/Cy3 Labeling

On each array in our transcriptome analyses, the wild-type sample was labeled with Cy3, and the osgi sample was labeled with Cy5. Therefore, we had to evaluate the dye effects using the same rice 44K Agilent array independently. First, we collected five sets of dye-swap microarray data that had been obtained previously with the same experimental protocol. See the details of samples in Supplemental Table 5 online. In this series of experiment, there were five sets of two treatments (leaf 0 min versus leaf 60 min after cutting, leaf 0 min versus leaf 30 min after cutting, root 0 min versus root 30 min after cutting, panicle 0 min versus panicle 30 min after cutting, embryo with abscisic acid [ABA] treatment versus embryo without ABA treatment), and each dye-swap pair used the same treatment (A or B in Supplemental Table 5 online) for both Cy3 and Cy5. The simple box plot of the 20 microarray data suggests the lack of overall dye effect in the rice 44K Agilent chip microarray experiments when we used log2 of the processed data provided by the Agilent protocol as an intensity of gene expression (see Supplemental Figure 32 online). To do a statistical test for a per-gene dye effect, we further adopted two-way analysis of variance (ANOVA: without interaction) analysis for all the 27,201 genes simply using the R function, or aov. The two factors were in the two-way ANOVA model: Dye and Combo, where Dye has two levels (Cy3 and Cy5) and Combo has 10 levels (leaf 0 min – sample 1, leaf 30 min, leaf 0 min – sample 2, leaf 60 min, root 0 min, root 30 min, panicle 0 min, panicle 30 min, embryo with ABA, embryo with Moc). We included all data of obtained cy3 and cy5 intensity even if they are background levels. Therefore, each gene has 10 cy3 intensity data points and the corresponding 10 cy5 intensity data points. Obtained P values for the Dye effect (at the gene level) after two-way ANOVA were FDR corrected by another R function, or p.adjust (method = “fdr”). The minimum data of FDR-corrected P value among 27, 201 genes was 0.01062 (see Supplemental Data Set 2 online), which indicates that no gene among 27,201 genes exhibited any dye effects (FDR = 0.01). Otherwise, only nine genes among them exhibited some dye effects (FDR = 0.01) among 27,201 genes tested (see Supplemental Data Set 2 online). Furthermore, averaged log2 (cy3 intensity/cy5 intensity) ratios of the nine genes were within 0.5 and -0.5 (see Supplemental Figures 33 and 34 online). These possible dye effects do not affect any evaluations on all the conclusions from the field transcriptomes described in the text. Therefore, we are confident that we could assume there were no dye effects on our data in using our microarray protocols for the rice 44K Agilent array.

Plant Growth in the Field

Rice plants were grown in paddy fields at Tsukuba, Japan (Figure 2 and Supplemental Figure 5 online, 2005; Supplemental Figure 6 online, 2007; Figures 3 and 4M, 2008; Figures 4H to 4L, 2009) or in Sendai, Japan (2009) (Figures 4A to 4G) in pots containing nutrient solution (Makino et al., 1988) by water culture in an environmentally controlled greenhouse maintained with day/night temperatures of 25/20°C and 60% relative humidity under natural sunlight (Figure 4). In the paddy fields, plants were transplanted at one plant per 20 x 20 cm (2005) or per 15 x 30 cm (2007, 2008, and 2009). Major transplanting dates were May 18, 2005 (Tsukuba), June 16, 2008 (Tsukuba), and April 1, 2009 (Sendai). For the microarray (Figure 3) and fertility (Figure 4M) data, plants were transplanted into paddy fields on June 1, 8, 15, and 22 (2008); these dates were later than those in typical cropping seasons in Tsukuba. For yield-related traits (Figures 4H to 4L), plants were transplanted on May 19, 2009. Heading dates were recorded every 2 or 3 days and considered as the flowering dates. For agronomic traits, the means ± SD were used from 10 (Figure 4H) or nine (Figure 4I) randomly selected independent plants at each stage. In total, 40 (Figure 4H) or 36 (Figure 4I) plants in the field were measured. Nine plants with about average panicle numbers were measured for grain weight (Figure 4J), grain yield (Figure 4K), and fertility (Figure 4L). For each transplanting date, nine plants were tested for fertility under atypical conditions (Figure 4M).

Photosynthesis and Carbohydrate Analyses

Gas-exchange rates and chlorophyll fluorescence were simultaneously measured on the uppermost fully expanded leaves with a portable open gas-exchange system (LI-6400; Li-Cor) equipped with a Mini-PAM photosynthesis yield analyzer (Walz) as described (Hirotsu et al., 2004). Measurements were made at a photosynthetic photon flux density of 1200 μmol m\(^{-2}\) s\(^{-1}\), a leaf-to-air vapor pressure difference of 1.0 to 1.2 kPa, a leaf temperature of 25°C, an ambient CO\(_2\) partial pressure of 39 Pa, and intercellular CO\(_2\) partial pressures of 20 Pa and >60 Pa. The gas exchange parameters were determined (Genty et al., 1989), and the quantum efficiency of photosystem II and the level of thermal dissipation (i.e., the nonphotochemical quenching) were estimated (von Caemmerer and Farquhar, 1981). These measurements were recorded indoors from June 1st to June 3rd in 2009. Plants used for measurements were moved to the room in the morning, and measurements were completed by 16:00. Ambient light conditions on June 1 to 3, 2009 are shown in Supplemental Figure 7 online. After the measurements, leaf nitrogen and chlorophyll contents were measured (Makino et al., 1988). To determine the
carbohydrate contents, the uppermost fully expanded leaves were collected, weighed, and immediately frozen in liquid nitrogen at different time points (at 4-h intervals) on a clear day (on June 9th) and then stored at –80 °C until analysis. Ambient light conditions on June 9, 2009 are shown in Supplemental Figure 7 online. Suc and starch contents were measured with an F-Kit (R-Biopharm) in accordance with the manufacturer’s instructions.

**Metabolomic Analysis**

Rice plants were grown in paddy fields at Tsukuba, Japan. The transplanting date was June 19, 2009. Leaves were sampled from the paddy field every 2 h from 07:00 on August 24, 2009 to 07:00 on August 25, 2009 and were immediately frozen in liquid nitrogen. Ambient environmental conditions on August 24th to the 25th in 2009 are shown in Supplemental Figure 7 online. A leaf was used as the sample for metabolomic analysis. All samples were derived from different plant individuals. Leaves were lyophilized and were crushed in a Mixer Mill (MM300; Retsch). Five milligrams (dry weight) of powdered tissue was used. Extraction with 1 mL of 80% aqueous methanol containing 0.1% formic acid, 33.6 mM lidocaine, and 1.72 μM 10-camporphosphoric acid, and the subsequent sample preparation, were fully automated. Metabolite contents were quantified by means of widely targeted metabolomics (Sawada et al., 2009) with 545 sets of retention time (RT) and multiple reaction monitoring (MRM) conditions, which were optimized for quantification of ~700 compounds by tandem quadrupole mass spectrometry coupled with ultraperformance liquid chromatography (Waters). Some of the RT-MRM sets detected more than one metabolite [e.g., D-(−)-ribose and D-xylulose, which is indicated as 2i, D-(−)-ribose, D-xylulose in Supplemental Data Sets 3 and 4 online]. Details of the methods of sample preparation and metabolomic analysis have been given previously (Sawada et al., 2009). Statistical analyses of metabolome data were performed using R. The significance of difference between the wild type (N8) and mutant (osgi) was tested by a paired t test. FDRs were calculated from the P values of the paired t tests (Benjamini and Hochberg, 1995). Differences in compound amounts between paired samples are represented in the difference plots (upper right of Supplemental Data Set 4 online). The estimated distributions of the compound amounts are shown in the density plots (bottom right of Supplemental Data Set 4 online).

**Accession Numbers**

Sequence data for Os-GI can be found in the GenBank database under accession number NM_001048755.1. Microarray data were deposited in the Gene Expression Omnibus under accession number GSE18685.

**Author Contributions**

T.I. designed this work, organized collaborations, performed most of experiments, and wrote the manuscript. M.M. helped to analyze the transcriptome data and worked with our database. H.I. performed complementation tests and mutant sequencing. M.Y.H. and Y.S. performed the metabolome analysis. A.J.N. analyzed the metabolome data. Y.S. and A.M. performed assays of photosynthesis and primary metabolism products and analyzed primary production in the microarray data. R.M. and Y.N. helped in the microarray analysis. M.Y. helped to screen the suppressor mutants. M.G. helped with genotyping for the association analysis.

**Supplemental Data**

The following materials are available in the online version of this article.

**Supplemental Figure 1.** qRT-PCR Analysis of Time Course of Expression of Hd3a, Hd1, and Os-GI in se5 Suppressors Mutants.

**Supplemental Figure 2.** osgi Mutations Identified in This Work Are Shown Schematically.

**Supplemental Figure 3.** Complementation Test.

**Supplemental Figure 4.** qRT-PCR Analysis of Circadian Clock–Related Genes in se5 Suppressors Mutants.

**Supplemental Figure 5.** Histograms of Flowering Time in the Field.

**Supplemental Figure 6.** Shift-of-Transplanting-Date Experiments for Flowering Time in the Field.

**Supplemental Figure 7.** Diurnal Changes in Ambient Environmental Conditions for Some of the Experiments.

**Supplemental Figure 8.** Average Calibrated Data for Core Circadian Clock–Related Genes in Rice.

**Supplemental Figure 9.** Average Calibrated Data for Other Circadian Clock–Related Genes in Rice.

**Supplemental Figure 10.** Average Calibrated Data for CO-like and COL9-like Genes in Rice.

**Supplemental Figure 11.** Histograms of Gene Numbers with the Time Points, at Which Expression Levels Were Changed Significantly by osgi-1.

**Supplemental Figure 12.** Heat Map of Pairwise Pearson’s Coefficients for Average Calibrated Data for Genes with Fitting Thresholds of <0.6.

**Supplemental Figure 13.** Mathematical Fittings for Cosine Curves.

**Supplemental Figure 14.** Examples of Regressed Waves to Average Calibrated Data of 27,201 Genes.

**Supplemental Figure 15.** Two-Dimensional Histograms for Peak Phases of the 24-h Period and Amplitudes of 13,338 Wild-Type Genes and 12,318 osgi Genes.

**Supplemental Figure 16.** Correlation Diagram of Peak Phases of a 24-h Period.

**Supplemental Figure 17.** Peak Phase Shifts for Each Time Point in osgi.

**Supplemental Figure 18.** Correlation Diagrams of a, b, and the Fitting Index between the Wild Type and osgi.

**Supplemental Figure 19.** Average Processed Data of Starch Synthesis–Related Genes in Rice.

**Supplemental Figure 20.** Average Processed Data of Starch Degradation–Related Genes in Rice.

**Supplemental Figure 21.** Average Processed Data of TCA Cycle–Related Genes in Rice.

**Supplemental Figure 22.** Average Processed Data of Calvin Cycle–Related Genes in Rice.

**Supplemental Figure 23.** Average Processed Data of Sucrose Metabolism–Related Genes in Rice.

**Supplemental Figure 24.** Average Processed Data of Oxidative Reaction of Pentose Phosphate Pathway–Related Genes with a Process of Conversion of Fructose 6-Phosphate to Glucose 6-Phosphate in Rice.

**Supplemental Figure 25.** Average Processed Data of Glycolysis I–Related Genes in Rice.

**Supplemental Figure 26.** Average Processed Data of Glycolysis II–Related Genes in Rice.

**Supplemental Figure 27.** Average Processed Data of Photochemical Reaction–Related Genes in Rice.

**Supplemental Figure 28.** Average Processed Data of Mitochondrial Electron Transfer–Related Genes in Rice.

**Supplemental Figure 29.** Average Processed Data of Phenylpropa- noid Pathway–Related Genes in Rice.
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Os-GIGANTEA Confers Robust Diurnal Rhythms on the Global Transcriptome of Rice in the Field

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