The effects of phytochrome-mediated light signals on the developmental acquisition of photoperiod sensitivity in rice

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Plants commonly rely on photoperiodism to control flowering time. Rice development before floral initiation is divided into two successive phases: the basic vegetative growth phase (BVP, photoperiod-insensitive phase) and the photoperiod-sensitive phase (PSP). The mechanism responsible for the transition of rice plants into their photoperiod-sensitive state remains elusive. Here, we show that the extremely early flowering mutant X61 is a nonsense mutant gene of OsHY2, which encodes phytochromobilin (PWB) synthase, as evidenced by spectrometric and photomorphogenic analyses. We demonstrated that some flowering time and circadian clock genes harbor different expression profiles in BVP as opposed to PSP, and that this phenomenon is chiefly caused by different phytochrome-mediated light signal requirements: in BVP, phytochrome-mediated light signals directly suppress Ehd2, while in PSP, phytochrome-mediated light signals activate Hd1 and Ghd7 expression through the circadian clock genes’ expression. These findings indicate that light receptivity through the phytochromes is different between two distinct developmental phases corresponding to the BVP and PSP in the rice flowering process. Our results suggest that these differences might be involved in the acquisition of photoperiod sensitivity in rice.

Flowering time plays a principal role in the regional adaptability of plants. In rice (Oryza sativa L.), a facultative short-day plant, flowering time is promoted under short-day length (SD), but is delayed under long-day length (LD). Development before floral initiation in rice is divided into two phases: the basic vegetative growth phase (BVP) and the photoperiod sensitive phase (PSP), and different rice varieties vary widely in the durations of the two phases1,2. During BVP, even varieties with strong photoperiod sensitivities do not respond to daylength1,2.

To date, several flowering time genes have been identified. Among these, Heading date 3a (Hd3a) and RICE FLOWERING LOCUS T 1 (RFT1), two orthologs of Arabidopsis FLOWERING LOCUS T (FT) that are known as florigen-like genes, are expressed in the vascular tissues, and their proteins of leaves move to the shoot apical meristem (SAM) through the phloem3-5. Upstream of Hd3a and RFT1, Heading date 1 (Hd1) and Early heading date 1 (Ehd1), two major floral signal integrators, process multiple signals6-7 originating from OsMADS50, OsMADS51, OsMADS56, Grain number, plant height, and heading date 7 (Ghd7)11, Ehd312, OsCOL413, Os-GIGANTEA (OsGI, an ortholog of Arabidopsis GIGANTEA [GI])14,15, Ehd2 (an ortholog of maize INDETERMINATE 1 [ID1], and also known as RID1 and OsID1)16-18 and Hdb19. Hd1 and Ehd1 are activated by OsGI and Ehd2, respectively14,16. In contrast, the expression of Ghd7, which encodes a CO, CO-LIKE, and TIMING OF CAB1 (CCT) motif-containing protein, is specifically upregulated in response to long-day (LD) conditions to repress Ehd1 expression11. The expression level of Ghd7 is determined by the coincidence of the
timing of gating and phytochrome-mediated light signals\textsuperscript{30}. Although many genetic factors that control flowering time in rice so far have been identified already, it remains unknown how rice developmentally acquires photoperiod sensitivity.

Recent studies in Arabidopsis thaliana, a long day model plant, have demonstrated the participation of a circadian clock in photoperiodic control of flowering time and have shown that its molecular base is composed of three major loops. This series of interlocked transcription–translation feedback loops constitutes the regulatory network of the circadian clock. The first loop is composed of the pseudo response regulator \textit{TIMING OF CAB EXPRESSION 1 (TOC1)}\textsuperscript{22} and two partially redundant Myb-like transcription factors, LATE ELONGATED HYPOCOTYL (LHY)\textsuperscript{22} and CIRCADIAN CLOCK ASSOCIATED 1 (CCA1)\textsuperscript{22}. In the morning, expression of \textit{LHY} and \textit{CCA1} represses \textit{TOC1} by binding to its promoter\textsuperscript{24}. The circadian accumulation of \textit{TOC1} in the evening then induces expression of \textit{LHY} and \textit{CCA1}. In the morning, the accumulation of \textit{LHY} and \textit{CCA1} also activates two \textit{TOC1}-related protein genes, \textit{PSEUDO RESPONSE REGULATOR 7 (PRR7)} and \textit{PRR9}\textsuperscript{20,26}, which subsequently repress \textit{LHY} and \textit{CCA1}. In the evening, \textit{GI} activates \textit{TOC1} expression, but in turn is negatively regulated by \textit{LHY}, \textit{CCA1} and \textit{TOC1}\textsuperscript{27,28}. In rice, several clock-related genes have been identified based on daily amplitude rhythms and homology searches, including \textit{OsLHY}, \textit{OsPRR1}, \textit{OsPRR37} (also known as \textit{Hd2}), \textit{OsPRR73}, \textit{OsPRR59}, and \textit{OsPRR95}\textsuperscript{22–31}. In addition, the flowering time genes of \textit{OsGI} and \textit{EF7} (also known as \textit{Hd17} and \textit{OsELF3-1}) have also been identified as clock-related genes\textsuperscript{13,16,32–38}.

It has recently been reported that the amplitudes of clock gene expressions are significantly reduced in the Arabidopsis phytochrome null mutant (\textit{phyABCDE})\textsuperscript{39}. It has also been reported that the cooperative interaction between the clock-related gene \textit{EARLY FLOWERING 3 (ELF3)} and \textit{Phytochrome} genes contribute to the maintenance of the clock gene expressions\textsuperscript{39}. These findings indicate that the phytochrome-mediated light signals play critical roles in the maintenance of clock oscillation and amplitude in Arabidopsis. On the other hand, only three relevant molecules have been identified in the rice genome: \textit{PHYA}, \textit{PHYB}, and \textit{PHYC}. The triple mutant \textit{phyABC} exhibits significantly reduced photoperiod sensitivity\textsuperscript{40}. Additionally, the loss of function mutant of the rice \textit{Se5} gene, an ortholog of the Arabidopsis \textit{HY1} gene, which encodes heme oxygenase that converts heme to biliverdin IX\textsubscript{a} (BV) in the phytochrome-chromophore biosynthesis pathway, eliminates photoperiod sensitivity due to the complete deficiency of phytochromobilin (PFB) synthesis that is essential for photo-interconversion between Pr and Pfr\textsuperscript{39,40}. Phytochromes and phytochrome-chromophore are thus significantly involved in photoperiod sensitivity in rice. Little is known, however, about the developmental stage-dependent effect of phytochrome-mediated light signaling on the oscillation of circadian clock genes in rice.

Our previous study indicated that the extremely early flowering of the mutant line X61 was caused by a complete loss of photoperiodic response due to a novel single recessive mutant gene, \textit{se13}. Since X61 harbored a 1-bp insertion in exon 1 of \textit{OsHY2}, we assumed that \textit{se13} is a mutant gene of \textit{OsHY2}, encoding PFB synthase, which is involved in the final step in the phytochrome-chromophore biosynthesis pathway\textsuperscript{41}. In that study, however, we were able to demonstrate only a strong possibility that the \textit{Se13} locus is identical to the \textit{OsHY2} locus based on linkage and subsequent sequence analysis. In this study, we verified that \textit{se13} in X61 is a nonsense mutant gene of \textit{OsHY2} by conducting a complementation test and phytochrome spectrum analysis, and that X61 and two \textit{Se13}-silenced lines exhibit repressed photo-morphogenesis caused by blinding of the red/far-red light signals. Subsequent expression analysis showed that the expression profiles of a few flowering time genes are influenced by the genotype at the \textit{Se13} locus on one level or another and differ between the BVP stage and the primary stage of PSP. These findings indicate that light receptivity through the phytochromes is different between two distinct developmental phases corresponding to the BVP and PSP in the rice flowering process.

**Results**

The \textit{Se13} gene is identical to the \textit{OsHY2} gene. X61 harbors a single base insertion (C) in exon 1 of \textit{OsHY2}\textsuperscript{41}, which exhibits 49% similarity to \textit{HY2} in Arabidopsis. We first conducted a complementation test of \textit{se13} to ascertain whether the \textit{Se13} locus is identical to the \textit{OsHY2} locus. Before the complementation test, we carried out a 3′-RACE (rapid amplification of cDNA ends) PCR for the \textit{Se13} transcripts and identified six different RACE products (Supplementary Fig. S1a and b). Using the ORF finder (NCBI; http://www.ncbi.nlm.nih.gov/projects/gorf/), we predicted their coding sequences to specify the single RACE fragment encoding the putative \textit{Se13} cDNA fragment of 906 bp (~34 kDa) length. We introduced this 906-bp cDNA into X61 under the control of a CaMV 35S promoter. The \textit{Agrobacterium}-mediated gene transformation was conducted according to a procedure\textsuperscript{41} optimized for the “Gimbozu” variety (WT). We tested the flowering time of two independently obtained transgenic plants (\textit{X61-RNAi} #1, #2) under a 14.5-h daylength (LD) and a 10-h daylength (SD). Consequently, the cDNA fragment (35S::\textit{Se13}) from WT fully rescued the photoperiod-insensitive phenotype of the transgenic plants under LD conditions (Fig. 1a–c). In Arabidopsis, \textit{HY2} is a downstream gene of \textit{HY1} in the phytochrome-chromophore biosynthesis pathway\textsuperscript{43,44}. In rice, \textit{Se5} has been shown to be an ortholog of \textit{HY1}\textsuperscript{45}. Knowing this, we checked the flowering time of an \textit{Se5}-deficient mutant (\textit{se5Se13}) and a double mutant (\textit{se5se13}); we found that both mutants flowered at the same time as did \textit{se13} mutant (\textit{Se5se13}), under not only SD but also LD conditions (Supplementary Fig. S2). These results indicate that \textit{Se13} is a downstream gene of \textit{Se5}. Next, we measured spectrophotometrically detectable phytochrome\textsuperscript{46} in X61 to see if \textit{se13} affects its concentration. In WT, crude extracts from etiolated seedlings exhibited a typical red/far-red reversible spectrum of phytochrome A. In X61 under the same conditions, the phytochrome signal was undetectable (Fig. 1d, left). When the extract was prepared from a 10-fold larger quantity of tissues, however, a small signal was observed (Fig. 1d, right).

Taking these findings together with the results of the complementation test and the red and far-red light response test, we concluded that the \textit{Se13} locus is identical to \textit{OsHY2}, which is similar to \textit{Arabidopsis HY2}, which encodes PFB synthase, and \textit{se13} of X61 is a leafy mutant allele that retains a weak ability to respond to red and far-red light.

**Molecular characterization of \textit{Se13}**

In order to further characterize the \textit{Se13} gene, we produced \textit{Se13}-silenced plants with an RNAi-silencing vector pANDA\textsuperscript{46}. The effects of \textit{Se13}-silencing were evaluated using two \textit{T\textsubscript{1}} lines derived from two independently obtained \textit{T\textsubscript{0}} plants that were heterozygous for the transgene (\textit{Se13}-silenced gene). The expression of \textit{Se13} was strongly silenced in the two \textit{T\textsubscript{1}} lines (\textit{Se13-RNAi} #1, \textit{Se13-RNAi} #3) as compared to WT (Fig. 2b). Subsequently, we investigated the effects of light and dark conditions on the seedling growth of \textit{Se13-RNAi} #1, #3, X61, and WT (Fig. 2a, d). In the dark, all lines exhibited elongated coleoptiles and third leaves (Fig. 2a, d). In white light, seedling elongation was not inhibited in \textit{Se13-RNAi} lines, but was inhibited in \textit{X61} and WT. In third leaf length and coleoptile length, significant differences were observed among the lines (Fig. 2d): third leaf lengths in \textit{Se13-RNAi} #1, #3, X61, and WT were 14.8 ± 1.96 cm, 14.2 ± 2.10 cm, 12.5 ± 1.05 cm, and 10.06 ± 1.271 cm, respectively, and coleoptile lengths in \textit{Se13-RNAi} #1, #3, X61, and WT were 0.96 ± 0.09 cm, 0.91 ± 0.12 cm, 0.93 ± 0.1 cm, and 0.44 ± 0.05 cm, respectively. Thus, the degree of light inhibition in X61 was
intermediate between that in the Se13-RNAi and that in WT, supporting the earlier finding that se13 in X61 is a leaky mutant gene. Interestingly, at the three-leaf stage, the growth of T1 seedlings homozygous for the transgene was arrested; their leaves turned white and blighted, and eventually withered. It has been demonstrated previously that rice has only three different phytocrome genes (phyA-C) serving as red/far-red light receptors, and that mutations in the phytocrome-chromophore biosynthesis pathway affect all phytocrome species. On the other hand, phytocrome deficiency itself does not induce a lethal phenotype; the phyA-C triple mutants and the se5 (oshy1) mutant, in which the chromophore biosynthesis pathway is blocked by loss-of-function of Se5 (OsHY1), continue to grow until flowering. To determine the mechanistic relationship between the loss-of-function of the Se13 (OsHY2) gene and the termination of rice growth at the three-leaf stage, we examined the chlorophyll contents of Se13-RNAi #1, #3, X61, and WT. We further measured the contents of three precursors in the tetrapyrrole pathway, protoporphyrin IX (Proto), Mg-protoporphyrin IX (Mg-Proto), and Mg-protoporphyrin monomethyl ester (Mg-ProtoMe) (Fig. 2c). The chlorophyll a and b contents in Se13-RNAi #1 and #3 were significantly lower than those in X61 and WT (Fig. 2e). No significant differences in chlorophyll a and b contents between X61 and WT were observed, although X61 showed slightly lower levels than WT. In contrast, the contents of Mg-Proto and Mg-ProtoMe, both of which are metabolites in the chlorophyll biosynthetic pathway, did not significantly differ among the four lines. Compared to WT, however, Se13-RNAi #1, #3, and X61 accumulated abundant Proto (Fig. 2f).

Analysis of the effect of se13 on the duration of BVP. To estimate the transition day from BVP to PSP in X61 and wild-type (WT), we conducted a photoperiod transfer treatment, starting with a long daylength (LD; 24-h) and shifting to a short daylength (SD; 10-h) according to the model of Ellis et al. with slight modifications. In the transfer treatments, 13 pots for each line were initially kept under a 24-h daylength. The days to flowering of both X61 and WT were constant in plants that had been transferred early, but gradually increased with the length of time that had passed before the transfer. Using this analytical model, we successfully estimated the duration of BVP and the degree of PS expressed by regression coefficient (b) of days to flowering on transfer time during PSP under SD conditions (Fig. 3a). First, we started the photoperiodic transfer treatment with a 14.5-h daylength to create LD conditions. The duration of BVP in WT was calculated at 22.69 days. We could not calculate the duration of BVP in X61, because we could not identify the transition day of X61 from BVP to PSP due to its extremely weak photoperiod sensitivity (Supplementary Fig. S3). Under 24-h daylength conditions, however, we did observed that the flowering time of X61 was slightly delayed (Supplementary Fig. S4), confirming that X61 has extremely weak photoperiod sensitivity. Thus, we were able to estimate the transition day of X61 based on its behavior under 24-h daylength conditions. The BVP durations of X61 and WT were thus estimated at 23.7 ± 1.01 and 23.2 ± 0.87 days, respectively (Fig. 3b and c). The mutant allele se13 was found not to affect the duration of BVP at all, and the transition from BVP to PSP occurs just after 23 or 24 days after sowing (DAS). X61 exhibited a far smaller b value (0.346) than WT (0.934). Since b is an index of photoperiod sensitivity, this implies that se13 almost, but not completely, excludes photoperiod sensitivity.

Effect of se13 on the expression of other flowering time-related genes. We examined the expression levels of flowering time and clock-related genes in X61 and WT to identify potential downstream genes regulated by Se13 under LD conditions (14.5 h) at four different time points during development, 18, 22, 25 and 28 days after sowing (DAS). Based on the results of the photoperiod transfer treatment, we regarded the first two time points, at 18 and 22 DAS, as falling within of BVP, and the second two time points, at 25 and 28 DAS, as falling within the primary stage of early PSP (a few days after the termination of BVP). To identify developmental changes in the expression patterns of various genes related to the genotype at the Se13 locus, we measured the expression levels at these four time points. The genes tested are listed in Supplementary Table 1.

We first investigated the expression levels of Hd3a and RFT1, both of which encode the mobile flowering signal florigen. At 18 DAS, RFT1 and Hd3a exhibited a significantly higher expression level in X61 than in WT in the daytime, whereas at 22 DAS Hd3a exhibited a very low expression level in both X61 and WT, although RFT1 still exhibited higher expression (Fig. 4a and b). Because se13 of X61 is a leaky mutant, the repression of Hd3a at 22 DAS in X61 might be attributed to the weak ability to respond to red and far-red light. Therefore, RFT1 appeared to be solely responsible for promoting floral initiation in X61 under LD (Fig. 4a and b). Because Ehd1 was also upregulated only in X61 in the daytime, it was evident that Ehd1 promoted the expression of RFT1 in X61. We also examined the
Figure 2 | Molecular characterization of se13 plants. (a) Phenotypes of Se13-RNAi lines (#1, #3), X61, and WT (Nipponbare, NB) (from left to right). Plants were photographed at 12 days after sowing (DAS) under light (10 light/14 dark) and dark conditions. Scale, 5 cm (b) Expression levels of Se13 transcripts in WT (NB) and Se13-RNAi plants. Samples were harvested from the second leaf blades of seedlings 12 DAS at ZT 9. Means ± s.d. are shown (n = 10) (c) A schematic illustration of the metabolic pathway of tetrapyrrole adapted from previous reports. Abbreviations used are as follows: ProtoIX for protoporphyrin IX, Mg-Proto for Mg-protoporphyrin IX, Mg-ProtoMe for Mg-protoporphyrin monomethyl ester, Se5 for heme oxygenase 1, Se13 for phytochromobilin synthase, Chl1 for Mg-chelatase subunit D, and Chl9 for Mg-chelatase subunit I. Asterisks indicate quantified tetrapyrrole intermediates in this study. (d) Lengths of coleoptile and the third leaves of seedlings 12 DAS under light (10 light/14 dark) conditions. Se13-RNAi #1, #3, X61, and WT are represented in Figure 3d. (e) Chlorophyll content of Se13-RNAi #1, #3, X61 and WT. Means ± s.d. are shown (n = 10). (f) Tetrapyrrole intermediates content of Se13-RNAi #1, #3, X61 and WT. Means ± s.d. are shown (n = 10). Means followed by different letters are significantly different from that of WT [P < 0.05 according to Tukey’s honest significant difference test (a, b or a–c)].
expression levels of the genes upstream of Ehd1, viz. Ehd2 (also known as RID1 and OsI D1)16–18, Ehd315, OsMAD5S06, OsMAD5S19, OsMAD5S66, and OsCOL417. Among these genes, Ehd2 was specifically upregulated in X61, whereas the other genes exhibited no differences between X61 and WT (Fig. 4a and b). Interestingly, the expression of Ghd7 at 22 DAS was higher than that at 18 DAS without any differences between X61 and WT (Fig. 4a and b). In addition, all other genes not previously described maintained their expression patterns and levels regardless of the genotype at the Se13 locus (Supplementary Fig. S5). The results of this experiment thus indicate that Se13 repressed the Ehd1-dependent flowering pathway by downregulating the Ehd2 expression level under LD at 18 and 22 DAS, regardless of the high expressions of Hdl and Ghd7 genes.

At 25 and 28 DAS, Hdl3a was not upregulated in either X61 or WT, and the upregulation of RFT1 was again observed only in X61 (Fig. 4c and d). Although Ehd1 was also upregulated in X61, the expression of Ehd2 was not changed compared to that in WT during the daytime. On the other hand, reductions in the expression of Ghd7 were observed in X61 at both time points (Fig. 4c and d). Therefore, the upregulation of Ehd1 in X61 was attributed to the reduced expression of Ghd7 in addition to the lack of the modification of Ghd7 protein by phytochrome due to the weak ability of X61 to synthesize phytochromobilin during this period. In addition to Ghd7, the expression level of Hdl1 in X61 during the daytime was reduced at 25 and 28 DAS, in contrast to 18 and 22 DAS (Fig. 4c and d). To understand the effects of se13 on the upstream genes of Hdl1, we examined the expression levels of OsGI, OsP R R37 (Hdl2), OsP R R37, OsP R R59, and OsP R R55. In X61, the peak expression levels of OsGI and most of the OsP R R gene series were lower than those in WT (Fig. 5 and Supplementary Fig. S5). These results suggest that Se13 might function in the maintenance of the expression amplitudes of the circadian clock oscillator, and that the declined amplitudes of internal clock genes are the main cause of the reduced expression of Hdl1 in X61.

Relationships between the Se13 locus and the Hdl1 and Ghd7 loci.

Expression analysis showed that loss-of-function of the Se13 gene affected the expression levels of Hdl1 and Ghd7 particularly at 25 DAS. We investigated the relationship between the Se13 locus and the Hdl1 and Ghd7 loci using double mutants (se13h1, se13ghd7). Days to flowering (DTF) increased in the genotype of Se13h1, but not in the genotype of se13h1. The se13 gene prevented ghd7 from decreasing DTF; in the presence of Se13, however, ghd7 greatly increased DTF. The hd1ghd7 double-deficient mutants, however, did not flowered at the same time as the se13 recessive lines. These observations clearly demonstrate that, under LD, Se13 is involved in a genetic photoperiodic flowering pathway that includes Hdl1 and Ghd7 (Fig. 6).

Discussion

In the present study, we successfully verified that se13 in the extremely early flowering mutant X61 is a nonsense mutant gene of OsHY2 encoding phytochromobilin (PΦB) synthase, and that X61 is almost, but not completely, insensitive to red-light signals, as determined through a complementation test followed by spectroscopic and photomorphogenic analyses. Comparison of the expression profiles between WT and X61 suggests that the reduction of phytochrome-mediated light signals contributes to decrease the expressions of major photoperiod sensitive genes, Ghd7 and Hdl due to the declined amplitudes of internal clock genes, resulting to early flowering under long day condition.

In rice, phytochrome-mediated light signals play critical roles in controlling flowering time. The rice genome harbors three phytochrome molecules, PhyA, PhyB, and PhyC, and the presence of PhyB and PhyC is essential for the inhibition of flowering under LD conditions46. Also, the presence of the homo-dimer PhyA and the hetero-dimer PhyB-PhyC is assumed to be indispensable for the expression of photoperiod sensitivity. In a rice nonfunctional mutant for the Se5 locus encoding hem oxygenase in the phytochrome-chromophore biosynthesis pathway, Hdl1 expression level decreased under LD conditions at 28 DAS45, indicating that Hdl1 is regulated by phytochrome-mediated light signals. Moreover, Ghd7 is a flowering time repressor whose expression is determined by the coincidence of the timing of gating and phytochrome-mediated light signals45. In the present study, the diurnal expression of Hdl1 in X61 decreased under LD at 25 and 28 DAS (Fig. 4c and d), while those at 18 and 22 DAS were not different between WT and X61 (Fig. 4a and b). On the other hand, the Ghd7 expression at 25 DAS was reduced from the dusk to the beginning of the next light period, and that at 28 DAS was markedly reduced (Fig. 4c and d). In addition, we found that the Se13 locus interacts with the Hdl1 and Ghd7 loci, affecting flowering time under LD (Fig. 6). These results indicate that the inactivation of phytochromes caused by the deficiency of PΦB synthase brings about reduced expressions of Hdl1 and Ghd7 along with the developmental growth. Interestingly, although the Ghd7 expressions at 18 and 22 DAS were not different between WT and X61, that at 18 DAS was lower than that at 22 DAS (Fig. 4a and b). Matsubara et al.12 reported that Ghd7 expression is developmentally regulated: the gene activity is highest over the two weeks after germination and then gradually decreases to a basal level. Therefore, the up-regulation of Ghd7 at 22 DAS was not observed in the present study. However, this discrepancy might be due to the different experimental conditions, such as the genotypes and the developmental stages.
Figure 4 | Diurnal expressions of flowering time genes at 18, 22, 25 and 28 DAS. Expression profiles (a) at 18 DAS (b) at 22 DAS (c) at 25 DAS (d) at 28 DAS. Y-axis indicates investigated gene expression levels. Plants were grown under LD conditions. Transcription levels were observed every 3 h. Measurements were repeated three times using three biological replicates. Average values ± s.d. are shown. RUBQ2 is a ubiquitin gene used for the normalization of expression levels. LD, long day. ZT, Zeitgeber time (ZT 0: beginning of light period). White and black bars indicate light and dark periods respectively. A two-tailed Student’s t-test tested the difference between two means: **0.05 < P < 0.01; *P < 0.01.
Figure 5 | Diurnal expressions of circadian clock genes at 18, 22, 25 and 28 DAS. Expression profiles (a) at 18 DAS (b) at 22 DAS (c) at 25 DAS (d) at 28 DAS. Y-axis indicates investigated gene expression levels. Plants were grown under LD (14.5-h light/9.5-h dark) conditions. Transcription levels were observed every 3 h. Measurements were repeated three times using three biological replicates. Average values ± s.d. are shown. RUBQ2 is a ubiquitin gene used for the normalization of expression levels. LD, long day. ZT, Zeitgeber time (ZT 0: beginning of light period). White and black bars indicate light and dark periods respectively. A two-tailed Student’s t-test tested the difference between two means: **0.05 < P < 0.01; *P < 0.01.
DAS is consistent with previous report. However, we do not have a clear answer to the question of why significant differences in Ghd7 expression were not seen between X61 and WT at 18 and 22 DAS. It is considered that a weak light signal might be enough to induce the Ghd7 expression, or that other unknown factors associated with the circadian clock might be involved in its up-regulation. This question remains to be explored in future studies.

In Arabidopsis, the amplitude of rhythmic oscillation/luminosity (pCCA1::LUC2) in the phytochrome null mutant (phyABCDE), which was completely insensitive to red-light signals, was greatly reduced compared to that in the WT\(^6\). To maintain the oscillation of clock genes, ELF3 integrates phytochrome-mediated light (red light) signals by binding to the PRR9 promoter sequence in a LUX-dependent manner\(^7\). Although Nagano et al.\(^{30}\) indicates that not all clock components were conserved between Arabidopsis and rice, some clock-associated genes, such as OsCCA1 and the OsPRR-series including OsTOC1/OsPRR1, OsZTLs and OsLUX, as well as OsGI are highly conserved\(^29\). At 25 and 28 DAS, the peak expression levels of OsGI and most of the OsPRR gene series were decreased in X61, while those at 18 and 22 DAS were not different between WT and X61 (Fig. 5). Hayama et al.\(^{14}\), Shibaya et al.\(^{32}\) and Lin et al.\(^{33}\) reported that OsGI and OsPRR37 (Hd2) regulate Hd1 and Ghd7 expressions, respectively. Given this along with the expression profiles of Hd1 and Ghd7, we conclude that the phytochrome-mediated light signals are essential for the amplitude of the normal circadian oscillation, and that the decreased amplitudes of OsGI and OsPRR37 are the major causes of the loss of photoperiod sensitivity in X61. However, the diurnal expression peak of OsGI in WT at 25 DAS seemed to be specifically 6 h earlier than those at the other three time points (Fig. 5). It is unclear why the peak timing of OsGI is around ZT6 only at 25 DAS, or whether this peak shift is biologically significant event. In this study, however, we found the significant down-regulation of OsGI expression at 25 and 28 DAS in X61. Although our current understanding of the circadian clock in rice is not sufficient to explain how the clock is regulated, we regard the down-regulation of the peak expression levels of OsGI and most of the OsPRR gene series as a trigger for the down-regulation of Hd1 and Ghd7. Further analyses are necessary to resolve this matter.

We demonstrated a distinct differences in gene expression profiles based on phytochrome-mediated light signals between the earlier time points (18 DAS and 22 DAS) and the later time points (25 DAS and 28 DAS), establishing the earlier time points in one developmental stage and the later time points in another. Daylength transfer experiments showed that the first and second developmental stages were likely to correspond with the duration of BVP and PSP, respectively. In a narrow interpretation, the duration of PSP is defined as an effective growth stage of long-day dependent floral repression, while the duration of BVP is defined as a non-effective growth stage of long-day dependent floral repression. Therefore, the physiological differences in the responsiveness to phytochrome-mediated light signal might contribute to the differences of developmental stages (Fig. 7). In addition, the expression amplitudes of circadian clock genes were clearly different between in the BVP and the PSP (Fig. 5). These results suggest that the contribution of phytochrome (red light) signals to the circadian clock oscillations might be, along with developmental growth, the main means by which the

**Figure 6** Effect of the se13 mutant gene on flowering time and its effect in combination with non-functional Ghd7 or Hd1 alleles observed under LD conditions. Mean values of 300 plants and error bars representing ± s.d. are shown. Means followed by different letters are significantly different from that of WT [P < 0.05 according to Tukey’s honest significant difference test (a–d)].

**Figure 7** A proposed model for the photoperiodic control of rice flowering in long day conditions. (a) Se13, which encodes phytochromobilin synthase, represses Ehd2 expression in BVP and activates Ghd7 and Hd1 expressions via the circadian genes OsPRR37/Hd2 and OsGI during PSP, respectively. (b) Under LD conditions, expressions of Ehd1 and RFT1 are suppressed until a late stage of development (around 70 DAS)\(^6,26\). This is caused by the suppression of Ehd2 expression during the early developmental stage corresponding to BVP. After developmental phase transition to PSP, the expressions of Ehd1 and RFT1 are suppressed by Hd1 and Ghd7 expressions mediated by the circadian clock. Thus, Se13 delays flowering in rice under LD conditions. Pointed arrows indicate the upregulation of a gene; blunt-ended arrows indicate the downregulation of a gene.
long-day dependent floral repression is acquired during developmental acquisition of the floral repression.

It is noteworthy that at 18 and 22 DAS, the expression of RFT1 in X61 was upregulated despite the high expressions of Ghd7 and Hd1 (Fig. 4a and b). According to previous reports, Ghd7 and Hd1 proteins require PHYB to exercise their flowering-inhibiting functions. It is therefore considered that in X61, Ghd7 and Hd1 proteins were not able to produce their usual effect due to the lack of sufficient phytochrome-mediated light signals. Furthermore, the expressions of Eth2 and Eth1 were highly upregulated in X61 (Fig. 4a and b). Since Eth2 (RID1, OsID1) is a positive regulator of Eth1-dependent floral repression, this results in the upregulation of Eth1 expression promoted by Eth2, in addition to the light signal’s being of insufficient strength to mediate Ghd7 and Hd1 proteins. Although previous studies have demonstrated that Eth2 (RID1, OsID1) mRNA is more abundant in younger leaves than in older ones, obvious up-regulations at 18 and 22 DAS were observed in X61 leaves, which were completely opened (Fig. 4a and b). At 25 and 28 DAS, Eth2 expression in X61 was not changed compared to WT during the daytime (Fig. 4c and d). These findings indicate that the reductions in older leaves at 18 and 22 DAS are regulated by phytochrome-mediated light signals. Future studies will help to clarify why the changes in expression profiles in Eth2 (RID1, OsID1) are associated with the transition between developmental phases.

In the present study, X61 showed normal development until seed maturity, although the Se13-silenced plants were lethal (Fig. 2a). In Escherichia coli and Arabidopsis, mutants deficient in ferrochelatase (FC), which catalyzes Proto-to-heme conversion, became lethal due to over-accumulation of Proto. However, Proto was significantly accumulated in both Se13-silenced lines and X61. Since X61 shows normal development until seed maturity, Proto accumulation is considered not to be a direct cause of the lethality of the Se13-silenced lines (Fig. 3i). Instead, the excessive reduction in the amount of chlorophylls in Se13-silenced lines is the most likely cause of the lethality although there were no significant reductions in the amounts of Mg-Proto and Mg-Protopheme in Se13-silenced lines compared to WT. Red light is known to promote the greening process in etiolated seedlings by dramatically accumulating the protoclorophyllide oxidoreductase, PorA and PorB, which catalyze the conversion of protoclorophyllide into chlorophylls, suggesting that PorA and PorB might not be accumulated in Se13-silenced lines. Moreover, under white light, plant height and coleoptile elongation of Se13-silenced seedlings were not inhibited; rather, they were comparable to those of WT seedlings grown in the dark (Fig. 2, a and d). It is well known that PHYB plays a major role in the inhibition of seedling elongation via red light signaling. These results indicate that Se13-silenced plants completely lose red light photosensory capabilities. In the heme branch of the phytocrome-chromophore biosynthesis pathway of Arabidopsis, there are two enzymatic reactions mediated by heme oxygenase (HY1) and PFB synthase (HY2) that are involved in phytochromobilin biosynthesis in the plastid. In higher plants, heme oxygenase is governed by small gene families and gene repeats, whereas PFB synthase is governed by a single gene, in contrast to the significance of the phytocrome signals (SALAD-DB, http://salad.dna.aicr.crs.go.jp and ref. 43). The Pew1 and pew2 (HY1 and HY2 orthologs) double mutants in N. plumbaginifolia showed lethality at an early stage of development. The normal metabolic process of phytochromobilin biosynthesis is thus indispensable to the growth of the plant.

Growth retardation in the HY2-deficient Arabidopsis mutants is generally less than that in HY1-deficient mutants. It is known that PFB synthase is highly conserved among many plant species; in addition, all the known PFB synthase mutants are leaky, and a small deletion(s) and a simple base change(s) in an exon seldom leads to severe growth retardation. HY2 is categorized as one of the ferredoxin (Fd)-dependent bilin reductases (FDBRs), which requires ferredoxins (Fds) as electron donors for double bond reductions, and the PFB synthesis step was catalyzed mostly efficiently by an HY2: BV-AtFd2 heterodimeric complex. Site-directed mutagenesis analyses in the predicted Fds docking site of HY2 residues demonstrated that all mutants still retained the ability to bind BV. Using 3'-RACE PCR on the Se13 transcripts, we identified six different RACE products (Supplementary Fig. S1a and b). Although X61 harbors a 1-bp insertion in exon 1 of OsHY2, other transcript variants, such as transcript c-type, might redundantly function as counterparts in forming the OsHY2: BV-Fd heterodimeric complex. This may be the reason why X61 retains the ability to respond to red and far-red light unless it disturbs the formation of the OsHY2: BV-Fd heterodimeric complex. On the other hand, we used the common sequences of six RACE fragments to create the Se13-silenced plants. It is therefore considered that Se13-silenced transgenic plants could be obtained through the potent disruption of translation of truncated functional proteins (Supplementary Fig. S1a). Thus, we postulate that complete inactivation of red/far-red perception due to inability to absorb red light because of a complete deficiency of phytochromobilin (PFB) synthesis caused the severe reduction in chlorophyll content; consequently, the Se13-strongly-silenced plants exhibited serious damages (lethality) on plants.

It is well known that rice plants do not respond to the photoperiod during BVP. Many previous studies have identified a large number of flowering time genes and demonstrated that photoperiod sensitivity is determined by a coincidence of internal clock oscillations and external light signals. However, there have been not been any clear answers as to how the clock and light signals are integrated with the mechanism responsible for the developmental acquisition of photoperiod sensitivity. Although it remains unknown why rice plants do not respond to the photoperiod during BVP, the phenomena reported here is expected to help us uncover how the light signaling (red light) adjusts circadian oscillation and regulates the photoperiodic repression of flowering time under long day condition during PSP. Furthermore, this developmental transition corresponds to sexual maturation in most species, including insects, birds and mammals. Thus, our findings will also provide valuable information toward understanding sexual maturation and other photoperiod-dependent developmental processes, such as migration, hibernation, sexual behavior, and resoring of sexual organs.

### Methods

**Plant materials.** The extremely early flowering time mutant line X61 and its original variety Gimbou (WT) were used. Gimbou is a japonica rice variety that had been cultivated in Japan at least since the 1940s and perhaps for as long as 100 years. X61 is a mutant line that was induced by X-ray irradiation of WT seeds. In addition, se5 and se5se13 were used. se5 is an extremely early flowering time mutant line that was induced by gamma-irradiation of the japonica variety Norin-8, which is genetically close to WT, whereas se5se13 is a double mutant line that was developed as a cross between X61 and se5. The single mutant line, hd1, harbors a photoperiod-insensitive allele with the transposable element mPing inserted at the intron region of Hdt. The single mutant line, gh2, harbors a photoperiod-insensitive allele at the E1 locus, which is identical to the Ghd7 locus. se1hd1 and se1gdh7 are double mutant lines that were developed as crosses between X61 and hd1, and between X61 and gh2, respectively.

**Growth conditions.** Plants were grown in growth cabinets with environmental controllers (LPH-240SP; Nippon Medical & Chemical Instruments Co., Ltd, Osaka, Japan) at 70% humidity and 400 ppm CO2 concentrations under either SD conditions of daily cycles of 10 h light at 30°C or under LD conditions, consisting of 24 h light and 10°C. Under LD conditions, consisting of 14.5 h light and 9.5 h dark. Fluorescent white light tubes (400–700 nm, 100 μmol·m−2·s−1) were used as the artificial light source. **Photoperiodic transfer treatment.** According to the analytical model of Ellis et al. and Nishida et al., we performed a non-linear regression analysis using a data set obtained from the photoperiodic transfer treatments with SigmaPlot software, ver. 12 (Cranes Software International Limited, India). We set two daylength conditions: the short daylength (SD) was 10 h, and the long daylength (LD) was 24 h. Germinated seeds were sown on field soil in 5-cm square pots and covered with granulated soil.
Seeds were thinned to four plants per pot at 14 DAS. The plants were grown with daily cycles of 10 h of light at 30 °C and 14 h of dark at 25 °C for the SD condition, or continuous light at 30 °C for the LD condition. Fluorescent white light tubes (400–700 nm, 100 µmol m−2 s−1) were used as the artificial light source. Thirty pots for each line were initially kept under LD conditions. Seven days after sowing, one pot per line was transferred to the SD conditions. Once a pot had been transferred, it was kept under SD conditions until flowering. One pot per line was transferred at each of the following time points: 0, 7, 14, 21, 28, 35, 42, 49, 56, 63, 70, and 77 DAS.

Plasmid construction and transformation. The full-length cDNA was isolated by PCR using the primer pair to conjugate BamHI and ScaI sites at the 5′ and 3′ ends respectively, with primer pair forward, 5′-GGGAGAGAAGCCATGACGCCGCGGGCT-3′ and reverse, 5′-AGGCAAGATTTATGCTTGTTGCGCAT-3′. PCR products were sub-cloned into a plasmid vector pSKI+ and were introduced into the pMLH173 plant expression vector. The Agrobacterium tumefaciens (AHA101 strain)-mediated method was used for the transformation of the full-length cDNA of Se13 into X610 for the complementation test. For the silencing of Se13, 5′ UTR of Se13 was isolated by PCR using the primer pair forward, 5′-CACCCTAACCCTTGATGATTGGTAC-3′ and reverse, 5′-AAGGAGATTTGATCCCGAGAAAGGCGG-3′. The PCR products were sub-cloned into pENTR/DEST TOPO cloning vector (Life Technologies Inc., Carlsbad, CA, USA) and were introduced into the pANZA vector by means of an LR clonase reaction. Then, the Agrobacterium tumefaciens (LBA4404 strain)-mediated method was used for the transformation of the 3′ UTR of Se13 into the variety Oryza sativa japonica cv Nipponbare which is generally similar to Gisouzo.

High-throughput quantitative RT-PCR (qPCR). Total RNA was isolated with TriPure Isolation Reagent (Roche, Ltd., Basel, Switzerland) and treated with DNase I (Takara Bio Inc., Otsu, Japan). Precisely 1.5 µg total RNA was used for first-strand cDNA synthesis using the Transcriptor Universal cDNA Master with random primer (Roche Ltd.), cDNA that had been preamplified (for 18 cycles) with a TaqMan preamp master mix kit (Life Technologies Inc.) was used for qPCR after five-fold dilution with TE buffer (Teknova Inc., Hollister, CA). Precisely 10 nl of pre-amplified cDNA per sample was used for the quantitative analysis of gene expression performed with TaqMan Universal PCR Master Mix (Applied Biosystems Inc., Foster City, CA, USA) using gene-specific primers and probes. The melting curve analyses were performed in advance to quantify the PCR efficiency and to test correlations between different regular qPCR. All the samples attained the critical value of R 2 > 0.97 and 0.99 for 15°C (1.1). Forty-eight gene assays and cDNA samples were loaded into separate wells on a 48-plex fluorescent real-time PCR system (Fluidigm Corp., South San Francisco, CA, USA). The qPCR was run on the Biomark HD system (Fluidigm Corp.; 10 min at 95°C for activation of the hot-start enzyme, followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 70°C for 20 s, and elongation at 60°C for 60 s). Relative RNA expression for each gene in a sample was calculated according to the delta–delta Ct method. All assays were replicated three times using different plant samples.

Spectroscopic measures of photophyton. Measurements were taken according to methods described previously. Photophytons were extracted from the shoots of seven-day-old etiolated seedlings and partially purified with an ammonium sulfate precipitation as described in the literature. The absorption difference spectra of photophytons in the extract were measured with a U3310 spectrophotometer (Hitachi Technologies, Tokyo, Japan). All difference spectra were obtained by averaging the results from three photophyton preparations by red and near-infrared far-red irradiation. Samples were kept (600 nm) or far-red (730 nm) light was supplied from the excitation light of a RF-5300PC fluorescence spectrophotometer (Shimadzu Co., Ltd., Kyoto, Japan) using a light guide. All procedures were performed under a dim green safety light.

Measurement of chlorophyll content. Samples of fresh leaves were taken from two-week-old seedlings. At least two seedlings were sampled in each replication, and the samplings were conducted three times for measurement. Extraction and measurement of chlorophyll a and b content was performed according to methods described previously. The absorption difference spectra of chlorophyll in the extracts were measured with a spectrophotophotometer (Bioespcc 1660; Shimadzu Co., Ltd.).

Quantification of Protopx, Mgppto, and MgpptoM. Plant material (50–100 mg) was weighed and frozen in liquid nitrogen, then ground using a 2-mL sample tube grinding apparatus (Yasu-kikai Corp., Osaka, Japan). The powdered samples were suspended with 1 mL of acetone chilled at −20°C and centrifuged at 10,000 x g (4°C) for 10 min. The supernatant was mixed with 0.1 mL of acetone chilled at −20°C and 30°C and measured in triplicate at 665 and 649 nm following the procedures described (482). The absorbance values were converted into the correspondent standards purchased from Frontier Scientific Inc. (Logan, UT, USA). HPLC conditions were performed according to methods described previously.
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Author contributions

Y.Y. designed and performed the majority of the experiments. T.Y. and X.Q. conducted the identification and cloning of Se13. T.A., N.M. and T.K. performed molecular characterization of Se13. H.S., H.N. and Y.O. performed photoperiod transfer treatment and data analysis. H.S., K.Z., H.K. and S.T. conducted spectroscopic measurements of phytochrome. H.L., M.T. and Y.O. provided the X61 plant. Y.Y., H.S., T. Tsukiyama., T. Tanisaka. and Y.O. wrote the manuscript. All authors reviewed the manuscript.

Additional information

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