Genetic interaction involving photoperiod-responsive $Hd1$ promotes early flowering under long-day conditions in rice

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Although flowering in rice has been extensively investigated, few studies focused on genetic interactions. Flowering evaluation of two recombinant inbred line (RIL) populations involving photo-insensitive rice cultivars, Bengal and Cypress, and a weedy rice accession, PSRR-1, under natural long-day (LD) conditions, revealed six to ten quantitative trait loci (QTLs) and a major QTL interaction. In addition to the validation of several previously cloned genes using an introgression lines (IL) population of PSRR-1, a few novel QTLs were also discovered. Analysis of the marker profiles of the advanced backcross lines revealed that $Hd1$ allele of PSRR-1 was responsible for the photoperiodic response in the near-isogenic lines (NILs) developed in both cultivar backgrounds. Based on the phenotypic and genotypic data of the NILs, and NIL mapping population and the transcript abundance of key flowering pathway genes, we conclude that $Hd1$ and its interaction with a novel gene other than $Ghd7$ play an important role in controlling flowering under LD conditions. Our study demonstrates the important role of genetic interaction that regulates flowering time in rice and the need for further investigation to exploit it for breeding adaptable rice varieties.

Flowering time is a complex agronomic trait governed by both genetic factors and environmental cues$^{1,2}$. Variation in day length is an important environmental signal that regulates flowering in many plants$^3$. Based on the flowering response to day length variation, plants are classified as long-day (LD), short-day (SD) or day neutral plants. As an important cereal crop, rice is grown in places with wide variation in photoperiod all over the world$^4,5$. Despite the progress made in deciphering the molecular mechanisms involved in the flowering response, there are many fundamental unanswered questions due to the genetic complexity of this trait$^6$. Particularly, the molecular basis of the wide range of genetic variation, the coordination of the different downstream genes in regulatory networks, and the genes regulating these regulators are still not clear.

In rice, two independent flowering pathways have been recognized: $HEADING\ DATE\ 1$ ($Hd1$)-dependent pathway and $EARLY\ HEADING\ DATE\ 1$ ($Ehd1$)-dependent pathway$^7-10$. $Hd1$, an ortholog of $CONSTANS$ ($CO$) of Arabidopsis, promotes flowering under SD conditions but strongly represses flowering under LD conditions through regulation of the expression of $Hd3a$$^7,11$. On the other hand, $Ehd1$ promotes flowering under both SD and LD condition, but its effect is stronger in promoting flowering under SD condition by activating $Hd3a$ and its paralog $RFT1$$^9,10,12$. Both $Hd3a$ and $RFT1$ encode the mobile flowering signal proteins, which are essential for flowering initiation$^{13}$. $Ehd1$ is both positively and negatively regulated by a number of genes$^{14-22}$. Among these, $Ghd7$ is an important member of the flowering pathway that regulates plant height, heading date, and grain number$^{16}$. It delays flowering under LD by repressing $Ehd1$ transcription.

Diversity in flowering time in rice varieties is largely due to presence of diverged alleles of the flowering genes and their interactions$^{12,23-28}$. $LH8$ encoding a CCAAT-box-binding transcription factor with $Hd1$-binding activity...
delayed flowering by repressing the expression of Ehd1\textsuperscript{29}. Similarly, the physical interaction between *Heading date Associated Factor 1* (HAF1), a C3HC4 RING domain–containing E3 ubiquitin ligase, and *Hd1* influenced photoperiodic flowering response through regulation of *Hd1* accumulation\textsuperscript{30}. *Ghd7*, a key floral repressor gene with major influence on rice yield\textsuperscript{16}, was reported to influence the function of *Hd1* in delaying or promoting flowering under long-day condition\textsuperscript{31}. The binding of the protein complex formed by the CCT domain of Ghd7 protein and the transcription activating domain of Hd1 protein to the regulatory region of Ehd1 led to its repression and florigen genes under LD condition\textsuperscript{32}. Another study indicated that the adaptation to specific agroclimatic region and yield potential depended on the combinations of *Ghd7*, *Ghd8*, and *Hd1* in rice varieties\textsuperscript{33}. Since the time of transition from the vegetative to flowering stage is vitally important for maximizing productivity, elucidation of the new genetic determinants and their interactions controlling this transition is essential to breed new high yielding rice varieties adapted to a specific cropping season or agroclimatic region.

The current study focused on the elucidation of the genetic interaction involved in the flowering transition in response to photoperiod using unique genetic materials such as recombinant inbred line (RIL) and introgression line (IL) populations, and near-isogenic lines (NILs) developed from crosses involving two photo-insensitive cultivars and a weedy rice accession. We discovered that the *Hd1* from the weedy rice accession in a cultivated rice background exhibited late flowering under LD condition. We further demonstrated that early flowering and photo-insensitivity in weedy rice was due to genetic interaction between *Hd1* and a novel gene other than *Ghd7* on chromosome 7.

### Results

#### QTL mapping for heading date in BR and CR RIL populations.

All three parents were photo-insensitive, but the hybrids of the BR (Bengal x PSRR-1) and CR (Cypress x PSRR-1) crosses were highly photosensitive in the natural field environment. Although the difference in mean days to heading (DTH) between both populations was around 10 days, the range was wide in each population with some transgressive segregants flowering earlier and later than either parent (Supplementary Fig. S1). In both populations, the distribution was skewed toward earlier flowering. A majority of RILs flowered within 70–100 and 80–130 days in the BR and CR populations, respectively\textsuperscript{34}.

Ten QTLs were detected on 7 chromosomes accounting for 58% of phenotypic variation in the BR-RIL population (Table 1, Figs 1, and S2). There were 2 QTLs each on chromosomes 2, 7, and 12 whereas chromosomes 1, 3, 6, and 11 harbored only one QTL. A wide range of variation was observed with respect to the magnitude of additive effects and percentage of the phenotypic variation explained by each QTL. The 'Bengal' and 'PSRR-1' alleles were responsible for increased DTH in case of five QTLs each. For the largest effect QTL on chromosome 6, and 11 harbored only one QTL. A wide range of variation was observed with respect to the magnitude of additive effects of 'Bengal' allele. 'Phenotypic variation (%)' explained by each QTL. 'Source of allele increasing the trait mean.' ‘Estimate of the total phenotypic variation explained by the QTL from a multiple QTL model fit in QTL Cartographer\textsuperscript{35}.'

| QTLs    | Marker interval | Physical size of QTL (Mb) | Position\(a\) | LOD | AE\(b\) | PVE\(c\) | Increasing effect\(d\) |
|---------|----------------|---------------------------|---------------|-----|---------|---------|----------------------|
| qHD1\textsuperscript{29} | RM8278-RM8134 | 2.278 | 128.9 | 3.6 | 3.94 | 4.1 | Bengal |
| qHD2-1\textsuperscript{RA} | RM29-RM341 | 9.032 | 58.3 | 6.0 | −4.93 | 6.4 | PSRR-1 |
| qHD2-2\textsuperscript{RA} | RM112-RM250 | 0.761 | 126.2 | 3.6 | 3.26 | 3.0 | Bengal |
| qHD3\textsuperscript{RA} | RM3203-RM3372 | 0.659 | 3.0 | 3.6 | −4.87 | 3.1 | PSRR-1 |
| qHD6\textsuperscript{RA} | RM3431-RM4924 | 9.728 | 45.9 | 9.2 | −5.79 | 8.5 | PSRR-1 |
| qHD7-1\textsuperscript{RA} | Rec-RM214 | 6.721 | 46.0 | 28.0 | 13.16 | 31.0 | Bengal |
| qHD7-2\textsuperscript{RA} | RM22134-RM248 | 0.591 | 102.4 | 6.3 | 4.93 | 5.9 | Bengal |
| qHD11\textsuperscript{RA} | RM224-RM144 | 0.608 | 109.3 | 3.2 | 3.17 | 2.6 | Bengal |
| qHD12-1\textsuperscript{RA} | RM1208-RM3483 | 1.612 | 1.0 | 3.1 | −3.14 | 2.7 | PSRR-1 |
| qHD12-2\textsuperscript{RA} | RM28661-RM17 | 1.558 | 88.5 | 3.8 | −3.73 | 3.8 | PSRR-1 |
| Total\(e\) | | | | | | | 58.3 |

**Table 1.** Quantitative trait loci for heading date in BR-RIL population detected using a composite interval mapping procedure. ‘QTL peak position on the linkage map. ‘Additive effects of ‘Bengal’ allele. ‘Phenotypic variation (%),’ explained by each QTL. ‘Source of allele increasing the trait mean.’ ‘Estimate of the total phenotypic variation explained by the QTL from a multiple QTL model fit in QTL Cartographer\textsuperscript{35}.’
QTL mapping in IL population of ‘PSRR-1’ in ‘Bengal’ background. Evaluation of a genome-wide IL population of ‘PSRR-1’ developed in ‘Bengal’ background indicated that 92% of ILs had DTH similar to the recurrent parent (RP) (Supplementary Fig. S4). Three ILs with introgressed segments of chromosome 7 flowered earlier than ‘Bengal’ and nine ILs with ‘PSRR-1’ segments from chromosomes 2, 3, 6, 8, 10, and 12 exhibited late heading compared with RP (Supplementary Table S1). When overlapping of introgressed segments was analyzed, six genomic regions responsible for increasing DTH and two genomic regions for decreasing DTH were identified (Fig. 1). The coincident QTLs located on chromosomes 3, 6, and 7 in both populations could be validated in these ILs. In addition, a few QTLs present in either BR (qHD2-1, qHD7-1) or CR populations (qHD8) were also confirmed. Among all these ILs with significantly different DTH compared to RP, the chromosome 6 IL was extremely late and photosensitive with the largest effect. However, it was detected as a minor QTL in both RIL populations. For both
QTLs on chromosome 7, ‘Bengal’ alleles increased DTH, which was consistent with the observation that the ILs harboring this region showed early heading compared to ‘Bengal’.

Based on the physical location of the cloned flowering genes and the molecular markers, we could locate several of them on the linkage map. Those overlapping with the QTLs were $Ehd4$ ($Hd2$) on chromosome 3, $Hd1$ on chromosome 6, $Ghd7$ ($Hd4$) and $DTH7$ on chromosome 7, and $Ehd3$, $DTH8$ ($Hd5$ or $Ghd8$) on chromosome 8 (Fig. 1).

**Effect of photoperiod on DTH on parents and photosensitive NILs.** Both BRNIL-20 and CRNIL-58 showed strong photosensitivity (PS) with initiation of flowering in 160–165 days. Molecular marker profiles of these lines indicated that both NILs had a ‘PSRR-1’ segment on chromosome 6 (RM225–RM4924 in BRNIL-20 and RM8225–RM5371 in CRNIL-58) (Supplementary Fig. S5). Based on the physical map locations of the flanking markers in both NILs and of $Hd1$, we concluded that $Hd1$ was located on the introgressed PSRR-1 segments of both NILs but not $Hd3a$ and $RFT1$. Using the principle of substitution mapping, the location of $Hd1$ was narrowed down to the RM3431–RM4924 and RM8225–RM4924 regions in BRNIL-20 and CRNIL-58, respectively (Supplementary Fig. S5). The QTL results showing the peak position of this QTL in both BR and CR-RIL populations at RM3431 (8.74 Mb) and RM8225 (9.31 Mb), respectively (Fig. 1), provided further evidence regarding $Hd1$ involvement.

The staggered planting experiment in greenhouse revealed that DTH was similar on all planting dates for the parents, but both NILs took a much longer time (>140 days) for flowering compared to parents under LD condition (Fig. 2). DTH peaked for plantings done in month of April and then gradually decreased each month, with the lowest in July. The exposure to LD conditions most likely delayed flowering in these NILs. Upon exposure to SD (10 h day length) in greenhouse, flowering could be induced (Supplementary Fig. S6). Flowering response was studied in ‘Bengal’, BRNIL-20, and its $F_1$ under LD condition. The $F_1$ and NIL did not flower 105 d after planting in greenhouse (Supplementary Fig. S7). Monitoring of heading date indicated that DTH of $F_1$s between the RP and their corresponding NIL was extremely late and photosensitive. The $Hd1$ alleles behaved in additive manner in cultivated rice background.

**Sequence variation in $Hd1$ gene and $Hd3a$ promoter.** ‘Cypress’ and ‘Bengal’ $Hd1$ sequences were identical, but the ‘PSRR-1’ allele differed from both cultivars with a 123 bp deletion, a 36 bp insertion, a 2 bp insertion, and several SNPs (Supplementary Fig. S8). The 123 bp deletion in first exon was present in both ‘PSRR-1’ and

| QTLs | Marker Interval | Physical size of QTL (Mb) | Position | LOD | $AE_b$ | $PVE_c$ | Increasing effect$^d$ |
|------|----------------|--------------------------|----------|-----|--------|---------|------------------------|
| $qHD1^{CR}$ | RM265-RM5389 | 0.536 | 136.3 | 2.5 | −4.11 | 2.5 | PSRR-1 |
| $qHD3^{CR}$ | RM3203-RM3372 | 0.659 | 3.0 | 20.5 | −14.57 | 29.1 | PSRR-1 |
| $qHD5^{CR}$ | RM1366-RM3322 | 1.346 | 25.2 | 3.6 | −4.76 | 3.7 | PSRR-1 |
| $qHD6^{CR}$ | RM276-RM8225 | 3.079 | 56.3 | 3.1 | −5.05 | 3.7 | PSRR-1 |
| $qHD7^{CR}$ | RM3555-RM172 | 1.671 | 114.5 | 10.1 | 9.09 | 13.7 | Cypress |
| $qHD8^{CR}$ | RM1376-RM1111 | 1.605 | 24.8 | 5.1 | −6.21 | 6.8 | PSRR-1 |

Table 2. Quantitative trait loci for heading date in the CR-RIL population detected using a composite interval mapping procedure. $^a$Peak position of the QTL on the linkage map. $^b$Additive effects of ‘Cypress’ allele. $^c$Phenotypic variation (%) explained by each QTL. $^d$Source of allele increasing the trait value. $^e$Estimate of the total phenotypic variation explained by the QTL from a multiple QTL model fit in QTL Cartographer.41

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Figure 2. Response of ‘Cypress’, ‘Bengal’, ‘PSRR-1’, BRNIL-20, and CRNIL-58 to photoperiod in a greenhouse experiments. Planting of parents and NILs was staggered at different dates between early February and late July to expose the plants to different lengths of photoperiod. Standard deviations are indicated by the error bars.
BR-RIL population (Supplementary Table S2). The effects were negligible for interactions involving qHD6 BR promoter region. But there were 18 SNPs and a 12 bp insertion in ‘PSRR-1’ compared to ‘Cypress’ and ‘Bengal’.

Hd3a lack, was present in both ‘PSRR-1’ and ‘Nipponbare’. ‘Bengal’ and ‘Cypress’ sequences are identical for the binding domains like ‘Nipponbare’ but a CCT domain, which photo-insensitive cultivars ‘Bengal’ and ‘Cypress’ compared to ‘PSRR-1’ and ‘Nipponbare’. All three parents had B-Box type zinc finger zinc

‘Nipponbare’. Analysis of cDNA and deduced amino acid sequence revealed truncated amino acid sequences in genes (Fig. 4) revealed that

OsLhy level for Ehd2, OsGI, Hd6, ETR2 genes such as

NILs compared to ‘PSRR-1’ under both SD and LD. Expression pattern of flowering pathway genes.

Segregation of the Hd1 allele in the NIL F2 mapping population. Using primers flanking the 123 bp deletion in Hd1, we confirmed the presence of ‘PSRR-1’ Hd1 allele in CRNIL-58 and BRNIL-20. In the case of Cypress x CRNIL-58 cross, the F2 population could be classified into three groups: early (<90d), intermediate (91–130 d), and late (>130 d) (Supplementary Fig. S10A) and segregation of these three groups fit into 1:2:1 ratio. Genotyping of the population indicated that all early and late heading plants were homozygous for ‘Cypress’ and ‘PSRR-1’ allele, respectively and the plants with intermediate DTH were heterozygous (Fig. 3B). In the ‘Bengal’ x BRNIL-20 F2 population (n = 600), we also confirmed the same pattern of phenotypic segregation (Supplementary Fig. S10B) and genotypic segregation in a pool of 10 randomly selected plants from each early, intermediate, and late flowering group of segregants (Fig. 3A).

Expression pattern of flowering pathway genes. An analysis of expression profile of five key flowering genes (Fig. 4) revealed that Hd1 expression was slightly higher in parents than NILs under SD compared to LD condition. Both NILs and ‘PSRR-1’ had higher accumulation of Hd1 transcripts compared to both parents under LD. The Ehd1 transcript level was very high in ‘PSRR-1’, ‘Bengal’, and ‘Cypress’ under SD but its expression was significantly reduced in NILs, ‘Bengal’, and ‘Cypress’ compared to ‘PSRR-1’ under LD. On the other hand, transcript level of HD3a and RFT1 was negligible in NILs under LD while Ghd7 expression was relatively lower in NILs compared to ‘PSRR-1’ under both SD and LD.

There were three different patterns of expression among the parents and NILs in other flowering pathway genes such as Ehd2, OsGI, Hd6, ETR2, and OsLhy (Supplementary Fig. S11). There was no difference in transcript level for OsLhy under LD, but it was reduced in NILs compared to parents under SD. The Ehd2 and Hd6 expression in both NILs was relatively lower than all parents under both SD and LD. While there was not much variation in transcript level of OsGI and ETR2 among all lines under SD, it was relatively higher under LD in both NILs compared to all three parents. The pattern of expression of ETR2 in LD was exactly opposite to that under SD, i.e. transcript abundance was more in NILs under LD compared to parents, whereas it was more in parents compared to NILs under SD condition.

Discovery of putative gene interaction. There were three significant digenic QTL interactions in the BR-RIL population (Supplementary Table S2). The effects were negligible for interactions involving qHD6 BR and two other QTLs on chromosome 2. But the most significant one was between qHD6 BR and qHD7-1 BR, with LOD score of 24. This interaction was validated using three advanced backcross introgression lines (Supplementary Table S3) and BR-RIL population (Supplementary Table S4) using markers linked to qHD6 BR and qHD7-1 BR. Those lines with homozygous PSRR-1 segments in both qHD6 BR and qHD7-1 BR flowered early, but homozygosity for PSRR-1 allele at only qHD6 BR resulted in late flowering and photosensitivity. Comparison of the marker profiles of the late and early flowering RILs revealed that all early flowering RILs were homozygous for the PSRR-1 allele at both RM3431 and RM214 (Supplementary Table S4, Supplementary Fig. S12).

The IL7-3 harboring the qHD7-1 BR flowered significantly earlier than the recurrent parent ‘Bengal’ under both LD and SD conditions (Supplementary Fig. S13), but it was more pronounced under LD. This observation

Figure 3. Segregation of Hd1 alleles in Bengal x BRNIL-20 F2 population (A) and Cypress x CRNIL-58 F2 population (B). ‘PSRR-1’ homozygotes were late (>130 days), ‘Cypress’ or ‘Bengal’ homozygotes were early (<90 days), and heterozygotes flowered within 91–130 days. In Cypress x CRNIL-58 F2 population, 1020 plants were evaluated for flowering. The Hd1 genotyping result of a sample of 10 plants from each early, intermediate, and late group in each population is shown.
was consistent with the results from QTL analysis that PSRR-1 allele at qHD7-1 BR was responsible for reducing the DTH. The F₁ between the IL7-3 and BRNIL-20 was intermediate in flowering and the segregation of early, intermediate, and late flowering plants in the F₂ population fit into 5:8:3 ratio (Supplementary Fig. S14). Based on digenic interaction model (Supplementary Fig. S15), early flowering occurred when both genes were in homozygous condition for the ‘PSRR-1’ alleles. The gene on chromosome 7 putatively interacting with Hd1 was having no effect in homozygous recessive or heterozygous condition. Since Ghd7 was present in the introgressed segment of IL7-3, we initially hypothesized Ghd7 as the candidate interacting with Hd1. Expression of Hd1, Hd3a, and Ghd7 under SD was reduced in IL7-3 compared with ‘Bengal’ (Supplementary Fig. S16). To confirm the interaction hypothesis, an early flowering segregant (#229) homozygous for ‘PSRR-1’ allele of both Hd1 and Ghd7 was selected from the F₂ population of the cross BRNIL-20 × IL7-3. If Ghd7 is the candidate, all F₃ progenies were expected to flower early. However, we noticed that 9 plants were extremely late and 21 plants were either early or intermediate flowering type suggesting single gene segregation. Both early and late flowering plants were homozygous for the ‘PSRR-1’ Ghd7 allele (Supplementary Fig. S17). The physical location of Ghd7 was at 9.2 Mb, whereas the QTL peak was near RM214 located around 12.7 Mb position. Expression analysis of both Hd1 and Hd3a in F₃ progenies of #229 indicated that even though the expression of Hd1 in both early and late plants was the same, higher expression of Hd3a was observed in early heading plants compared with late ones (Supplementary Fig. S18).

Discussion
Natural genetic variation has been exploited to decipher the genetic basis of flowering in response to photoperiod37. In this study, we used both RIL34 and IL35 populations developed from crosses involving cultivated and weedy rice, which allowed assessment of the magnitude of the QTL effects on phenotype as well as discovery of a genetic interaction. The increased power of the IL population to detect novel and more QTLs compared to RIL populations was due to reduced genetic noise resulting from the segregation of fewer QTLs at the same time. It is particularly valuable under circumstances when large effect QTLs are masked by complex genetic interactions. In addition to the validation of several previously cloned genes7,38,39, few novel QTLs were also discovered in this study (Fig. 1). A genetic background effect was clearly evident from the QTL results in both populations. For example, the QTL qHD7-1 CR with largest effect in the BR-RIL population was not detected in the CR-RIL population. Similarly, the QTL corresponding to the largest effect qHD3 CR in the BR-RIL population had negligible effect. Development of ILs of ‘PSRR-1’ in the ‘Cypress’ background and comparison with the BR-ILs will be helpful to investigate the reasons for such discrepancies.

Development of early maturing rice varieties is an important breeding objective. In this study, we discovered that weedy rice alleles could be useful to shorten the maturity duration (For example, qHD7 CR in CR population, qHD1 BR, qHD2-2 BR, qHD7-1 BR, qHD7-2 BR, and qHD11 BR in BR population). Particularly, the transfer of qHD7-1 from ‘PSRR-1’ to several genetic backgrounds could be exploited in breeding early maturing varieties for US rice growing environments.

Results from previous studies on genetic interaction in weedy rice40,41 were different from the present study demonstrating the variability in weedy rice populations and complex genetic interactions involving several
heading date genes and their variants. Weedy rice alleles at three QTLs - Se7.1, Se7.2, and Se8 inhibited flowering\(^{28}\). In the other study\(^{41}\), a nonfunctional \textit{Hd1} crop allele and a weed allele of a QTL \textit{qHD7S} increased the heading date, whereas a weedy \textit{Hd1} allele with crop allele at \textit{qHD7S} locus resulted in early flowering. But we demonstrated that early flowering was due to the combination of the weedy alleles of \textit{qHD7-1} and \textit{Hd1} in a cultivated rice background. Most of these studies used photosensitive parents, which is in sharp contrast to the use of photoinsensitive parents in this study.

The weedy rice accession and both rice cultivars used in this study were all day-neutral. However, ‘Nipponbare’ and ‘Kasalath’, which were used to clone \textit{Hd1}\(^{7}\), were photosensitive and weakly photosensitive, respectively. Strong photosensitivity response in BR and CR hybrids provided the first evidence for the genetic interaction. The \textit{Hd1} allele of ‘PSRR-1’ was functional like ‘Nipponbare’ due to the presence of CCT domain. Using the NILs developed in two genetic backgrounds, we demonstrated that the \textit{Hd1} allele of ‘PSRR-1’ was responsible for late flowering and photosensitive response. Although the effect of \textit{Hd1} was additive in NILs, strong photosensitivity and extremely late flowering observed in BR and CR hybrids could be due to the segregation of other genes influencing this trait. Since both RIL populations segregating for photoperiodic response involved non-contrasting parents, we hypothesized that gene interaction involving \textit{Hd1} was responsible for the photo-insensitivity in ‘PSRR-1’ and the gene interacting with \textit{Hd1} should be in homozygous condition.

Our results demonstrated the role of \textit{Hd1} and its genetic interaction in regulating flowering and photoperiodic response. We ruled out the \textit{Hd3a} promoter sequence variation regulating flowering\(^{22}\) because there was no difference in transcript level of \textit{Hd3a} among the parents irrespective of day length variation (Fig. 4). Although the transcript level of \textit{Hd1} in both NILs was adequate under LD, the expression of both florigen genes, \textit{Hd3a} and \textit{RFT1}, was negligible (Fig. 4). As the transcript levels of \textit{Ehd1} and \textit{Ghd7} in both NILs were comparable with their respective recurrent \textit{paitnis} under LD, the integrated transcription of these genes. However, introgression of \textit{qHD7-1} and \textit{Hd1} from ‘PSRR-1’ in Bengal’ background resulted in early flowering under LD due to upregulation of the \textit{Hd3a} gene (Supplementary Fig. S18). The observation that the early flowering \textit{F}_\text{i} individuals from the cross between \textit{Hd1 NIL} (BRNIL-20) and IL7-3 harboring \textit{qHD7-1}\(^{28}\) were homozygous for \textit{Hd1} allele of ‘PSRR-1’ provided further evidence for genetic interaction between \textit{Hd1} and an unknown factor in the \textit{qHD7-1}\(^{28}\) region.

The interaction of \textit{Hd1} with \textit{Ghd7} was previously reported to regulate photoperiodic flowering\(^{31,32}\). The flowering induction or suppression activity of \textit{Hd1} under LD was dependent on the \textit{Ghd7} allelic status\(^{31}\). Both studies\(^{31,32}\) demonstrated that the physical interaction between the CCT domain of \textit{Ghd7} and the transcription activation domain of \textit{Hd1} led to suppression of expression of \textit{Ehd1} and florigen genes \textit{Hd3a}/RFT1 under LD condition resulting in late flowering. Since \textit{Ghd7} was located in the \textit{qHD7-1}\(^{28}\) region, it was necessary to determine if \textit{Ghd7} or a new gene is interacting with \textit{Hd1} leading to early flowering response. We summarized below the evidences to support the involvement of a new gene other than \textit{Ghd7} in this newly discovered genetic interaction, which is an important finding of this study. It was hypothesized earlier that the gene involved in the interaction with \textit{Hd1} should be in homozygous condition. The phenotypic segregation of the \textit{F}_\text{i} population into 5 early:8 intermediate:3 late ratio in the cross between BRNIL-20 and IL7-3 (Supplementary Fig. S15) proved the above hypothesis. The occurrence of late flowering plants that were homozygous for both \textit{Hd1} and \textit{Ghd7} alleles of PSRR-1 also eliminates the possible involvement of \textit{Ghd7}. We further selected an early flowering \textit{F}_\text{i} plant #229 which was homozygous for the weedy \textit{Hd1} and \textit{Ghd7} alleles as well as for the markers (RM7121, \textit{Rc}, RM214, and RM5793) located in the introgressed \textit{qHD7-1}\(^{28}\) region. Instead of uniform early flowering response, which is expected if \textit{Ghd7} is involved in interaction, the \textit{F}_\text{j} progeny of the plant #229 segregated for flowering implying involvement of a new gene. Since the plant #229 still retained a large introgressed segment of PSRR-1 flanking \textit{Ghd7}, it is highly unlikely that recombination within the gene resulted in chimeric \textit{Ghd7} in such a small population (\textit{n} = 282). Despite similar transcript level of \textit{Hd1} in both early and late \textit{F}_\text{j} segregants of plant #229, expression of \textit{Hd3a} and \textit{Ghd7} was higher in the former compared to the later under LD (Supplementary Fig. S18). The differential transcript level of \textit{Ghd7} and \textit{Hd3a} in both early and late group of plants may be attributed to the new gene. Another evidence against the involvement of \textit{Ghd7} was based on the fact that \textit{Ghd7} (9.15 Mb position) is physically located far away from RM214 (12.78 Mb position), which was closely linked to the \textit{qHD7-1}\(^{28}\) (Fig. 1) and was demonstrated to interact with RM3431 (closely linked to \textit{Hd1}) in the BR-RIL population (Supplementary Table S4).

Although the physical interaction between \textit{Ghd7} and \textit{Hd1} was responsible for extreme late flowering under LD condition, Nemoto \textit{et al.} (2016)\(^{35}\) did not rule out involvement of other genes or other mechanisms for the photoperiod-dependent reversal of \textit{Hd1} function. Their study\(^{35}\) provided many clues for involvement of unidentified genes other than \textit{Ghd7} in suppression of \textit{Hd1} supporting our conclusion in this study. For example, \textit{Hd1} overexpressing \textit{Kita-ake} with a nonfunctional \textit{Ghd7} delayed flowering under both SD and LD. But in our study, weedy \textit{Hd1} allele and cultivar \textit{Ghd7} allele were present in extreme late flowering BRNIL-20 and CRNIL-S8. On the contrary, the IL7-3 with a nonfunctional \textit{Hd1} allele and the weedy rice segment harboring weedy allele of \textit{Ghd7} flowered significantly earlier than the recurrent parent under both SD and LD (Supplementary Fig. S13). It was also speculated that the interaction among the flowering genes was dependent on developmental stages of the plant\(^{32}\) and \textit{Hd1} may be interacting with gene (s) other than \textit{Ghd7} at the vegetative stage of rice. Considering the above facts, it is highly likely that a new gene, possibly the one hypothesized in our study, may be involved in interaction with \textit{Hd1} for reversing its role as a transcriptional activator leading to promotion of flowering under LD condition. However, it remains to be determined if the same gene in IL7-3 is responsible for both early flowering and interaction with \textit{Hd1}, thus warranting further investigation including cloning of \textit{qHD7-1}\(^{28}\).

Our results suggest the new gene as a missing link between \textit{Hd1} and florigen genes and may be functioning downstream of \textit{Hd1} involving a post transcriptional mechanism. We propose to integrate the role of this new gene in the flowering pathway in following ways (Fig. 5): (a) The new gene may interact with the \textit{Hd1} to regulate the florigen genes, (b) the new gene product may physically interact with \textit{Ghd7}/\textit{Hd1} complex to directly regulate
florigen genes or through Ehd1 since multistep regulation of the downstream genes is possible. In conclusion, our study not only demonstrates unlocking of the hidden genetic diversity underlying the flowering time variation in response to photoperiod in weedy rice, but also emphasizes the need to discover novel loci and their genetic interactions for rice improvement.

**Materials and Methods**

**Plant materials.** Two RIL populations were developed from the cross combinations, Bengal x PSRR-1 and Cypress x PSRR-1. The Bengal × PSRR-1 RIL population (named as BR) was composed of 198 individuals in the F_{7:8} generation, whereas the 'Cypress × PSRR-1 population (named as CR) included 174 RILs in the F_{8:9} generation. 'Bengal' and 'Cypress' are high yielding rice cultivars developed by the Louisiana Agricultural Experiment Station. PSRR-1 is a weedy rice accession with light green pubescent leaves, vigorous growth habit, straw-hulled medium grain, open panicles, high seed shattering, and intense seed dormancy. It was purified by selfing for two generations from the seeds collected from the Rice Research Station of the LSU Agricultural Center located at Crowley, Louisiana. Weedy rice is a conspecific form of cultivated rice (Oryza sativa L.), which has been a major constraint for rice production in the US and other parts of the world.

A population of 74 homozygous introgression lines (ILs) covering the entire PSRR-1 genome in 'Bengal' background was developed by three rounds of backcrossing followed by two generations of selling (Supplementary Fig. S19). Marker assisted selection was employed in each generation to speed up the development of ILs with fewer donor segments and the IL population was in the BC_{3}F_{3} generation. BRNIL-20 was a photosensitive NIL of PSRR-1 in 'Bengal' background which was developed in the same manner without employing marker-assisted selection. It was identified in the BC_{3}F_{3} generation. Both photosensitive NILs contained a single 'PSRR-1' introgression in chromosome 6 based on genotyping using polymorphic simple sequence repeat (SSR) markers.

Two F_{3} populations were developed by crossing BRNIL-20 and CRNIL-58 to their respective recurrent parents to determine if Hd1 is responsible for heading date variation. IL7-3 was an early flowering IL of PSRR-1 in 'Bengal' background which was developed from the cross between BRNIL-20 and IL7-3 and an F_{3} plant (229) homozygous for PSRR-1 Hd1 and Ghd7 allele was selected and was evaluated in the F_{3} generation for the heading date in greenhouse.

**Phenotyping and genotyping.** The parents and both RIL populations were grown at the Central Research Station of the LSU Agricultural Center in Baton Rouge, LA (30°20'51"N, 91°10'14"W). Planting was done in the middle of April to ensure exposure to natural long-day conditions. Each line was sown in a 2-m row of 20 plants with a row spacing of 20 cm. Standard cultural practices were followed. The heading dates in both mapping populations were recorded on five randomly selected plants from each line. 'Days-to-heading' was defined as the number of days from seeding to the first panicle emergence in each plant. Mean temperature and day length...
The IL population and parents were evaluated at the same location following the same planting plan and cultural practices as described above. The photosensitive NILs, parents, and F$_2$ populations from the crosses, Bengal x BRNIL-20 (n = 600) and Cypress x CRNIL-58 (n = 1020), were grown in field condition. The individual plants were classified as early, intermediate, and late when flowering occurred <90 days, 91–130 days, and >130 days, respectively. A sample of 200 F$_2$ individuals of the Cypress x CRNIL-58 cross was genotyped using the 

### Sequencing of $Hd1$ gene and $Hd3a$ promoter.

The entire genomic and cDNA sequences of $Hd1$ of 'PSRR-1', 'Bengal', and 'Cypress' were amplified from the genomic DNA and cDNA using Phusion High Fidelity DNA Polymerase (New England Biolab, MA) with primers listed in Supplementary Table S6. Similarly, the $Hd3a$ promoter and 5′ UTR region upstream of ATG (~1975 bp) was amplified from genomic DNA of 'PSRR-1', 'Bengal', and 'Cypress'. Primers (Supplementary Table S6) were designed based on the available reference genome sequences of the Nipponbare in the rice genome annotation database (http://rice.plantbiology.msu.edu/). The gel purified PCR products were first cloned into the pGEM-T Easy vector system I (Promega Corp., WI) and three independent products were sequenced at the Genomic Facility of Louisiana State University. Genomic DNA, CDS, and deduced protein sequences were aligned using the MegAlign module of the Lasergene genomics suite version 14.0 (DNASTAR, Madison, WI). The 123 bp deletion of $Hd1$ genomic sequence was targeted to distinguish the $Hd1$ alleles via PCR using a pair of primers under the following thermocycler profile: 95 °C for 5 min; 35 cycles of 94 °C for 45 s, 60 °C for 45 s and 72 °C for 1 min; and a final extension at 72 °C for 5 min.

The genome sequence of 'Bengal' and 'PSRR-1' available in our laboratory was used to develop SNP markers for the $Ghd7$ gene. The $Ghd7$ alleles of 'PSRR-1' and 'Bengal' were amplified using primers $Ghd7-{F/Ghd7-BN-R}$, respectively, using the following thermocycler profile: initial denaturation at 94 °C, 3 min; 35 cycles of 94 °C for 30 s, 60–65 °C for 30 s and 72 °C for 45 s; and a final extension at 72 °C for 5 min.

### Quantitative real-time polymerase chain reaction (qRT-PCR) analysis.

Seeds of parents (Cypress, PSRR-1, and Bengal), and NILs (BRNIL-20 and CRNIL-58) were sown in mid-April and mid-July for exposure to natural LD condition and SD conditions, respectively. Top leaves were sampled for gene expression analysis 55 days after sowing. Total RNA was isolated using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA), followed by treatment with TURBO™ DNA-free DNase (Invitrogen, Carlsbad, CA, USA) to remove possible genomic DNA contamination. Quality of total RNA was checked in a 1.2% formamide-denaturing agarose gel, and quantification was done using a ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, USA). First-strand cDNAs were synthesized using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, USA) in a reaction volume of 20 µl. Gene specific primers for qRT-PCR (Supplementary Table S6) were designed for known flowering pathway genes using Primer3Web (version 4.0.0) software (http://bioinfo.ut.ee/primer3). The expression level of these genes was determined using a MyiQ BioRad Single Color Real-time PCR Detection System following the protocol described earlier. Each 10 µl of PCR sample contained 5 µl of SYBR Green mix (Bio-Rad, Hercules, USA),
diluted cDNA, and 0.4 μM of forward and reverse gene specific primers. The expression of each gene in different RNA samples was normalized with the expression of an internal control gene, rice Actin1 (LOC_Os05g36290.1). Melt curve analysis was performed to check the specificity of the amplified product and fold changes in mRNA expression of each gene in different genotypes compared to PSRR-1 was calculated by a. Each CT (cycle threshold) value represented the average of three biological replicates with three technical replicates.

References

1. Putterill, J., Laurie, R. & Macknight, R. It’s time to flower: the genetic control of flowering time. Bioessays 26, 363–373 (2004).
2. Verhage, L., Angenent, G. C. & Imamink, R. G. Research on floral timing by ambient temperature comes into blossom. Trends Plant Sci. 19, 583–591 (2014).
3. Garner, W. W. & Allard, H. A. Effect of the relative length of day and night and other factors of the environment on growth and reproduction in plants. J. Agric. Res. 18, 553–606 (1920).
4. Izawa, T. Adaptation of flowering-time by natural and artificial selection in Arabidopsis and rice. J. Exp. Bot. 58, 3091–3097 (2007).
5. Song, Y. H., Shim, J. S., Kinmonth-Schultz, H. A. & Imaizumi, T. Photoperiodic flowering: time measurement mechanisms in leaves. Annu. Rev. Plant Biol. 66, 441–464 (2015).
6. Colasanti, J. & Coneva, V. Mechanisms of floral induction in grasses: something borrowed, something new. Plant Physiol. 149, 56–62 (2009).
7. Yano, M., Kojima, S., Takahashi, Y., Lin, H. X. & Sasaki, T. Genetic control of flowering time in rice, a short-day plant. PLoS ONE 10, e0130650 (2015).
8. Paterson, A. H., DeVerna, J. W., Lanini, B. & Tanksley, S. D. Fine mapping of quantitative trait loci using selected overlapping recombinant chromosomes, in an interspecies cross of tomato. Genetics 124, 735–742 (1990).
9. Yano, M., Kojima, S., Takahashi, Y., Lin, H. X. & Sasaki, T. Genetic control of flowering time in rice, a short-day plant. Plant Physiol. 127, 1425–1429 (2001).
38. Gao, H. et al. *Ehd4* encodes a novel and *Oryza*-genus-specific regulator of photoperiodic flowering in rice. *PLoS Genet.* **9**, e1003281 (2013).
39. Gao, H. et al. Days to heading 7, a major quantitative locus determining photoperiod sensitivity and regional adaptation in rice. *Proc. Natl. Acad. Sci. USA* **111**, 16337–16342 (2014).
40. Gu, X. & Foley, M. E. Epistatic interactions of three loci regulate flowering time under short and long day lengths in a backcross population of rice. *Theor. Appl. Genet.* **114**, 745–754 (2007).
41. Qi, X. et al. More than one way to evolve a weed: parallel evolution of US weedy rice through independent genetic mechanisms. *Mol. Ecol.* **24**, 3329–3344 (2015).
42. Linscombe, S. D. et al. Registration of 'Bengal' rice. *Crop Sci.* **33**, 645–646 (1993).
43. Linscombe, S. D. et al. Registration of 'Cypress' rice. *Crop Sci.* **33**, 355–355 (1993).
44. Wang, S., Basten, C. J. & Zeng, Z. B. Windows QTL Cartographer 2.5. Department of Statistics, North Carolina State University, Raleigh, NC (2011). http://statgen.ncsu.edu/qtlcart/WQTLCart.htm (accessed 29 May 2017).
45. SAS Institute Inc. SAS® 9.4 System Options: Reference, Second Edition. Cary, NC: SAS Institute Inc. (2012).
46. Eshed, Y. & Zamir, D. An introgression line population of *Lycopersicon pennellii* in the cultivated tomatoes enables the identification and fine mapping of yield-associated QTL. *Genetics* **141**, 1147–1162 (1995).
47. Karan, R., DeLeon, T., Biradar, H. & Subudhi, P. K. Salt stress induced variation in DNA methylation pattern and its influence on gene expression in contrasting rice genotypes. *PLoS ONE* **7**, e40203 (2012).
48. Livak, K. J. & Schmittgen, T. D. Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔCT method. *Methods* **25**, 402–408 (2001).

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Author Contributions
P.K.S. (Subudhi) conceived and supervised the complete study. R.T., T.D., C.C., R.K., J.O., and P.K.S. (Singh) conducted the experiment and analyzed the data. P.K.S. (Subudhi) wrote the manuscript. All authors read and approved the manuscript.

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