Hd3a, a Rice Ortholog of the Arabidopsis FT Gene, Promotes Transition to Flowering Downstream of Hd1 under Short-Day Conditions

Shoko Kojima 1,5, Yuji Takahashi 1, Yasushi Kobayashi 2,3, Lisa Monna 1,6, Takju Sasaki 4, Takashi Araki 2 and Masahiro Yano 4,7

1 Institute of the Society for Techno-innovation of Agriculture, Forestry and Fisheries, Tsukuba, Ibaraki, 305-0854 Japan
2 Department of Botany, Graduate School of Science, Kyoto University, Sakyo-ku, Kyoto, 606-8502 Japan
3 Core Research for Evolutional Science and Technology (CREST), Japan Science and Technology Corporation, Kawaguchi, Saitama, 332-0012 Japan
4 Department of Molecular Genetics, National Institute of Agrobiological Sciences, Tsukuba, Ibaraki, 305-8602 Japan

Heading date 3a (Hd3a) has been detected as a heading-date-related quantitative trait locus in a cross between rice cultivars Nipponbare and Kasalath. A previous study revealed that the Kasalath allele of Hd3a promotes heading under short-day (SD) conditions. High-resolution linkage mapping located the Hd3a locus in a ~20-kb genomic region. In this region, we found a candidate gene that shows high similarity to the FLOWERING LOCUS T (FT) gene, which promotes flowering in Arabidopsis. Introduction of the gene caused an early-heading phenotype in rice. The transcript levels of Hd3a were increased under SD conditions. The rice Heading date 1 (Hd1) gene, a homolog of CONSTANS (CO), has been shown to promote heading under SD conditions. By expression analysis, we showed that the amount of Hd3a mRNA is up-regulated by Hd1 under SD conditions, suggesting that Hd3a promotes heading under the control of Hd1. These results indicate that Hd3a encodes a protein closely related to Arabidopsis FT and that the function and regulatory relationship with Hd1 and CO, respectively, of Hd3a and FT are conserved between rice (an SD plant) and Arabidopsis (a long-day plant).

Keywords: Flowering time — QTL — Rice — Short-day plant.

Abbreviations: CAPS, cleaved amplified polymorphic sequence; CO, CONSTANS; FT, FLOWERING LOCUS T; Hd, heading date; LD, long-day; NIL, nearly isogenic line; PAC, P1-derived artificial chromosome; QTL, quantitative trait locus; RT, reverse-transcription; SD, short-day.

The nucleotide sequences reported in this paper have been submitted to the DNA Data Bank of Japan under accession numbers AB052941, AB052942, AB052943, AB052944, AB062675, and AB062676.

Introduction

The timing of the transition from vegetative to reproductive phase—floral transition—is important to ensure successful sexual reproduction of plants and is regulated by both endogenous and environmental factors. In Arabidopsis, genetic analysis using flowering mutants revealed multiple pathways that control the floral transition (Koornneef et al. 1998a, Koornneef et al. 1998b, Piñeiro and Coupland 1998, Simpson and Dean 2002). Photoperiod and vernalization pathways are involved in the response to environmental signals, such as long-day (LD) photoperiod and low temperature in winter, respectively. Autonomous and gibberellin pathways respond to endogenous signals whose nature is still unknown. Our understanding of the photoperiod pathway has made great progress through the identification and analysis of key genes (Reeves and Coupland 2000, Araki 2001). CONSTANS (CO) encodes a zinc finger protein and promotes flowering under LD conditions (Putterill et al. 1995). Overexpression of CO was sufficient to promote early flowering independent of photoperiod (Onouchi et al. 2000). FLOWERING LOCUS T (FT), which encodes a protein similar to phosphatidylethanolamine-binding protein, also promotes flowering under LD conditions (Kobayashi et al. 1999, Kardailsky et al. 1999), and is a direct target of CO (Samach et al. 2000). CO expression exhibits distinct circadian oscillation under short-day (SD) and LD conditions (Suárez-López et al. 2001). Suárez-López et al. (2001) suggested that the regulation of transcription by the circadian clock and, possibly, of protein stability by light conditions result in differential CO activity, which in turn results in different levels of FT mRNA under SD and LD conditions. Thus, CO acts between the circadian clock and promotion of flowering by FT.

Floral transition is promoted under SD conditions in rice. Flowering time (often referred to as heading date) is determined mainly by photoperiod sensitivity and duration of basic vegetative growth. Genetic analysis revealed that several genes are involved in the response to photoperiod in rice (Yokoo et al. 1980, Yamagata et al. 1986, Sano 1992, Yokoo and Okuno 1993, Tsai 1995). But little is known about how these genes...
regulate photoperiodic flowering at the molecular level. With the advance of molecular marker-based mapping, we performed quantitative trait locus (QTL) analyses for heading date. Heading date 1 (Hd1) to Heading date 14 (Hd14) were identified as QTLs for heading date in crosses between *japonica* cultivar Nipponbare and *indica* cultivar Kasalath (for review, see Yano et al. 2001). To understand the molecular mechanism of the control of heading in rice, we have tried to identify genes for these QTLs. So far, we have identified genes for *Hd1* and *Hd6* by map-based cloning. *Hd1*, a gene for a major QTL controlling photoperiod sensitivity, encodes a putative zinc finger protein that shows high homology to *Arabidopsis* CO (Yano et al. 2000). The Nipponbare allele at *Hd1* enhanced photoperiod sensitivity, promoting heading under SD conditions and delaying heading under LD conditions. These facts suggest that CO-like genes play an important role in photoperiodic control of flowering in both LD and SD plants, and raise an interesting question about whether their transcriptional regulation and downstream targets are conserved between LD and SD plants. *Hd6* encodes an alpha subunit of protein kinase CK2, and the Kasalath allele delays heading under LD conditions (Takahashi et al. 2001). The overexpression of CK2β caused early flowering in *Arabidopsis* (Sugano et al. 1999). These facts indicate that CK2 is involved in a photoperiod-dependent pathway in both species.

*Hd3a* was originally identified as a single QTL for heading date located on the short arm of chromosome 6 (Yamamoto et al. 1998). More recently, analysis of advanced backcross progeny and nearly isogenic lines (NILs) revealed two distinct genes, *Hd3a* and *Hd3b*, in the *Hd3* region (Monna et al. 2002). NIL(*Hd3a*), an NIL homozygous for the Kasalath allele at the *Hd3a* locus in the genetic background of Nipponbare, headed earlier than Nipponbare under SD conditions and headed at almost the same date as Nipponbare under LD and natural field conditions in Tsukuba, Japan (Monna et al. 2002). On the other hand, the Kasalath allele at *Hd3b* causes late heading under LD and natural field conditions, but not under SD conditions. The phenotypic effect of the Kasalath allele at *Hd3*—the promotion of heading under SD conditions—was observed in the presence of the Nipponbare allele at *Hd1* or *Hd2* (Lin et al. 2000). These results suggest that *Hd3a* could interact with *Hd1* and *Hd2* under SD conditions (Lin et al. 2000, Monna et al. 2002). In this study, we identified *Hd3a* by map-based cloning. *Hd3a* is a functional ortholog of *Arabidopsis FT* and promotes heading under SD conditions. Expression analysis using Nipponbare and an NIL with a loss-of-function allele of *Hd1* indicates that *Hd3a* functions downstream of *Hd1* within a genetic network regulating heading date in rice. These results suggest that the function of *Hd3a* and *FT* and the regulation of their expression by *Hd1* and CO, respectively, are conserved between rice (an SD plant) and *Arabidopsis* (an LD plant). This study also demonstrated a difference in the expression profiles of the key flowering time genes *Hd3a* and *FT* in response to daylength between rice and *Arabidopsis*.

### Results

**Identification of *Hd3a* candidate gene**

*Hd3a* has been roughly mapped on chromosome 6 (Monna et al. 2002). To determine its precise position, we performed further analysis using 2207 recombinant plants (Fig. 1A). Using cleaved amplified polymorphic sequence (CAPS) markers, we selected 10 plants in which recombination occurred in the vicinity of *Hd3a*. Progeny testing of each recombinant determined the position of *Hd3a*. We screened a P1-derived artificial chromosome (PAC) library of the Nipponbare genome and found that two PAC clones, P0046E09 and P0698G05, covered this region (Fig. 1B). We developed CAPS markers by using the nucleotide sequence of PAC clone P0046E09 to define the region of *Hd3a* more precisely. The position of *Hd3a* was delimited to a ~20-kb region between CAPS markers 25-5UL and CP59 (Fig. 1C).

In the region of *Hd3a*, GENSCAN (http://genes.mit.edu/GENSCAN.html) predicted four genes, cds25, cds26, cds27 and cds28 (Fig. 1C). Sequence cds25 showed high similarity to *Arabidopsis* FT (Fig. 1D), which promotes flowering under LD conditions. Of the other predicted genes, cds26 did not show significant similarity to any known protein, cds27 showed similarity to lipid transfer protein, and cds28 to acyl CoA synthase (data not shown). Reverse-transcription (RT)-PCR analysis showed that expression of cds25 was induced when plants were transferred from LD to SD conditions (Fig. 1E). Under LD conditions (16-h light), which does not induce heading, cds25 mRNA was not detected in either Nipponbare or NIL(*Hd3a*). When the plants were transferred from LD to SD conditions (10-h light), which induces heading, the expression of cds25 was detected in both lines at 6 d after the transfer and increased until at least 10 d. In contrast, the other predicted genes did not show SD-specific expression: cds26 mRNA was not detected, and cds28 mRNA was detected at the same level in both lines under SD and LD conditions; cds27 was expressed only in Nipponbare, under SD and LD conditions (data not shown). Therefore, we further analyzed cds25 as a likely candidate for *Hd3a*.

We examined the sequence of a cosmid clone, H3PZ1-1, which contains the region containing cds25 in Kasalath, and compared it with the corresponding Nipponbare sequence. Many sequence variations were found in the 4.2-kb genomic region (Fig. 1F). Among them, a 1-base substitution and a 2-base substitution were present in the coding region (Fig. 1G). The former was a synonymous substitution; the latter caused an amino acid change at the carboxyl end of the predicted protein: Asn in Kasalath and Pro in Nipponbare. RT-PCR analysis showed that the splicing sites were the same in both alleles (data not shown).

**Introduction of genomic DNA fragment containing cds25 into Nipponbare**

We introduced an 8.7-kb SpeI genomic fragment (Fig. 1C) carrying cds25 from Nipponbare or Kasalath into Nipponbare...
**Fig. 1**  Cds25 is a candidate gene for Hd3a. (A) Genetic linkage map showing the relative position of Hd3a on chromosome 6. The number of recombinant plants is indicated between markers. (B) Nipponbare PAC clones spanning the Hd3a region. (C) Detailed genetic and physical map of the Hd3a region. The candidate region is shown in the box. Black circles indicate the position of each CAPS marker. Horizontal arrows indicate the region of predicted genes. The 8.7-kb SpeI fragment was used in the complementation test of Hd3a. (D) Alignment of amino acid sequences of Kasalath cds25 and similar sequences in public databases. cds22, a similar gene upstream of Hd3a (designated RFT1, GenBank accession number AB062676); FT and TFL1, genes related to flowering time in *Arabidopsis* (Bradley et al. 1997, Ohshima et al. 1997, Kobayashi et al. 1999, Kardailsky et al. 1999). (E) RT-PCR analysis of the cds25 transcripts in Nipponbare and NIL(Hd3a) (top). Control PCR products were amplified with actin-specific primers (bottom). All plants were grown under LD conditions for 30 d and were then subjected to the indicated additional treatments. PCR was also done without template (no template) and with genomic DNA. The amplified fragments were electrophoresed and blotted onto a membrane, then hybridized with cds25 and actin probes. The number of PCR cycles was 22 for cds25 and 20 for actin. (F) Comparison of cds25 nucleotide sequences between Nipponbare and Kasalath. Hatched boxes indicate coding region. The vertical bar indicates base substitution. Black triangle and open triangle indicate insertion and deletion in Nipponbare compared with Kasalath. Transcriptional initiation site is indicated with an arrow. *We could not decide on the precise number of TA repeats in this region of either allele. (G) C-terminal region of Hd3a. Boxes indicate nucleotide and amino acid sequences that vary between Kasalath and Nipponbare.

by *Agrobacterium*-mediated transformation to verify the function of cds25. The regenerated plants (T₀) were grown under SD or LD conditions. The heading dates of transformants with the transgene from Kasalath or Nipponbare were earlier than those of plants with the vector alone, indicating that both transgenes can promote heading (Table 1).

There was a good correlation between the copy number of the transgene and the earliness of heading dates. For example, in a plant with seven copies of the Kasalath transgene, the first panicle appeared at 16 d after transplanting from agar to soil. This extremely early heading produced only a few spikelets, and seeds failed to mature. On the other hand, plants carrying a single copy of the Kasalath transgene headed between 34 and 38 d after transplanting. These plants had some mature seeds. Plants with two or three copies of the transgene showed an intermediate phenotype. It is likely that a large copy number of the transgene and/or a positional effect caused high levels of expression, which in turn resulted in extremely early heading, irrespective of photoperiod.

If we compare transformants (other than those heading extremely early that have multiple copies of the transgene), transformants with the Kasalath transgene tended to head earlier than those with the Nipponbare transgene. Table 2 shows the heading date of the T₁ progeny of transformants that carried a low copy number of the Kasalath or Nipponbare transgene. The self-pollinated progeny showed segregation of heading date under SD conditions. Plants with the transgene headed early, whereas those without the transgene did not. Cds25K10-2, the progeny of plants with a single copy of the Kasalath transgene, showed early heading. Cds25K2-1 and cds25K8-4, the progeny of plants with two and three or four copies of the Kasalath transgene, showed earlier heading. Cds25N4-1, the
**Table 1** Frequency distribution of days to heading in T<sub>1</sub> transgenic plants

<table>
<thead>
<tr>
<th>Days to heading&lt;sup&gt;c&lt;/sup&gt; under SD conditions</th>
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<tbody>
<tr>
<td>15</td>
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<tr>
<td>------</td>
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<tr>
<td>Exp. 1, SD&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Exp. 2, SD&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Exp. 3, SD&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Vector</td>
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</table>

*SD<sup>a</sup> and LD<sup>a</sup>, plants were grown under SD (10-h light) and LD (13-h light) conditions with a photon irradiance of 600–700 μmol m<sup>−2</sup> s<sup>−1</sup>.

*SD<sup>b</sup> and LD<sup>b</sup>, plants were grown under SD (10.5-h light) and LD (13.5-h light) conditions with a photon irradiance of 300–350 μmol m<sup>−2</sup> s<sup>−1</sup>.

*Days to heading<sup>c</sup> under SD conditions: number of days required from transplanting to soil to the appearance of the first panicle.

*Table 2** Frequency distribution of days to heading in T<sub>1</sub> transgenic plants

<table>
<thead>
<tr>
<th>Days to heading under SD&lt;sup&gt;−&lt;/sup&gt; conditions</th>
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<tr>
<td>15</td>
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<tr>
<td>------</td>
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<tr>
<td>Exp. 1, LD&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Exp. 2, LD&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>Vector</td>
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</table>

SD<sup>a</sup> and LD<sup>a</sup>, plants were grown under SD (10.5-h light) and LD (13.5-h light) conditions with a photon irradiance of 300–350 μmol m<sup>−2</sup> s<sup>−1</sup>.

Under this LD conditions, neither Nipponbare nor NIL(Hd3a) headed at 130 d after sowing. cds25K10-2, cds25K2-1, and cds25K8-4: T<sub>1</sub> lines transformed with pPZ-cds25K, pPZ-cds25N, and pPZ2H-lac vector (no insert). N, no panicle after 80 d from transplanting.

Under this LD conditions, neither Nipponbare nor NIL(Hd3a) headed at 130 d after sowing. cds25K10-2, cds25K2-1, and cds25K8-4: T<sub>1</sub> lines transformed with pPZ-cds25K, pPZ-cds25N, and pPZ2H-lac vector (no insert). N, no panicle after 80 d from transplanting.
Overexpression of sense and antisense transgenes of Hd3a in rice

We introduced a Kasalath Hd3a cDNA fragment into Nipponbare under the control of the cauliflower mosaic virus 35S promoter. Overexpression of Hd3a in Nipponbare caused early heading. The effect was stronger than that of the genomic Hd3a fragment whose expression was controlled under its own promoter. In most of the plants, the transition to reproductive phase occurred extremely early (Fig. 2A). These plants had few spikelets, which did not mature, probably because of insufficient vegetative growth. We also introduced antisense Hd3a cDNA of the Kasalath allele into NIL(Hd3a) (Fig. 2B). Some transformants with decreased levels of Hd3a transcript showed late heading (Fig. 2C). These results suggest that the level of the Hd3a transcript affects heading date.

Overexpression of sense and antisense transgenes of Hd3a in Arabidopsis

To test whether overexpression of Hd3a promotes flowering in an LD plant, we introduced the same sense overexpression constructs into Arabidopsis. Overexpression of Hd3a from Kasalath or Nipponbare resulted in extremely early flowering of T1 plants, as observed in those overexpressing FT (Table 3). There was no significant difference in phenotype between the Kasalath and Nipponbare transgenes, which differ by one amino acid at the carboxyl terminus (Fig. 1G), indicating that the proteins from both alleles are equally active in Arabidopsis (Table 3). We selected one homozygous line overexpressing Hd3a from Kasalath and examined the photoperiod response. As in the case of FT (Kobayashi et al. 1999, Kardailsky et al. 1999), plants overexpressing Hd3a showed photoperiod-independent early-flowering phenotype (Table 4).

Hd3a expression in Nipponbare and NILs

We compared expression levels of Hd3a in Nipponbare and NIL(Hd3a) grown under SD and LD conditions (Fig. 3). Hd3a transcripts were detected early and gradually increased with time under SD conditions (Fig. 3A). In NIL(Hd3a), Hd3a introduction of a Nipponbare genomic fragment containing cds26 or cds27, of which no transcripts were detected in NIL(Hd3a), into NIL(Hd3a) did not affect heading date (data not shown).

Table 3 Number of leaves in T1 transgenic Arabidopsis over-expressing FT or Hd3

<table>
<thead>
<tr>
<th>Transgene</th>
<th>Number of leaves</th>
<th>SD</th>
<th>Range</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>35S::FT</td>
<td>4.5</td>
<td>0.6</td>
<td>3–6</td>
<td>201</td>
</tr>
<tr>
<td>35S::Hd3a-K</td>
<td>4.1</td>
<td>0.6</td>
<td>2–6</td>
<td>223</td>
</tr>
<tr>
<td>35S::Hd3a-N</td>
<td>4.1</td>
<td>0.4</td>
<td>3–5</td>
<td>84</td>
</tr>
<tr>
<td>None</td>
<td>10.7</td>
<td>0.9</td>
<td>8–12</td>
<td>54</td>
</tr>
</tbody>
</table>

a Non-transgenic Columbia plants grown on 1/2× MS – 1.5% sucrose media.
Hd3a promotes flowering downstream of Hd1 in rice

mRNA was detected at 8 d after sowing, but Hd3a mRNA was barely detectable in Nipponbare until after that time. Under LD conditions, the amount of Hd3a transcript was remarkably reduced in all lines and did not increase even after heading (Fig. 3B). The expression level of Hd3a in NIL(Hd3a) was higher than that in Nipponbare under SD conditions, indicating that the Kasalath allele confers higher levels of expression.

In the process of delimitation of the Hd3a locus, we found an informative plant with a recombination point in the Hd3a region. We selected a homozygous recombinant plant (270-2) and a reference plant (270-5), which were derived from the F3 progeny of the recombinant. The region upstream of the transcriptional initiation site was homozygous for Kasalath allele in both lines; the downstream region was homozygous for the Nipponbare allele in 270-2 and for the Kasalath allele in 270-5 (Fig. 4B). Expression levels of Hd3a in these plants were examined to determine whether the 5′ promoter region confers the difference in the level of expression of Hd3a between Nipponbare and 270-2 (Fig. 4A). These results strongly suggest that the allelic difference between Kasalath and Nipponbare Hd3a is determined by the region downstream of the transcriptional initiation site.

Previous genetic studies suggested that Hd3a, which is active under SD conditions, acts downstream of Hd1 (Lin et al. 2000, Monna et al. 2002). FT in Arabidopsis acts downstream of CO (Kobayashi et al. 1999, Kardailsky et al. 1999) and is a direct target of the CO protein (Samach et al. 2000). To investigate whether Hd1 regulates Hd3a, we also quantified the expression levels of Hd3a in an NIL for Hd1 [NIL(Hd1)] (Fig. 3). In NIL(Hd1), the Nipponbare functional allele was replaced with a Kasalath allele whose product lacks the C-terminal region (Yano et al. 2000). NIL(Hd1) showed later heading than did Nipponbare under SD conditions. The expression levels of Hd3a were reduced in NIL(Hd1), indicating that the functional allele of Hd1 up-regulates the expression of Hd3a.

Diurnal oscillation of Hd1 and Hd3a mRNA abundance

In Arabidopsis, CO and FT transcript levels oscillate with distinct circadian rhythms (Suárez-López et al. 2001). We examined the diurnal rhythm of expression of Hd1 and Hd3a

Table 4 Photoperiodic response of transgenic Arabidopsis overexpressing FT or Hd3a

<table>
<thead>
<tr>
<th>Transgenic line</th>
<th>Photoperiod</th>
<th>Number of leaves</th>
<th>SD</th>
<th>Range</th>
<th>n</th>
<th>t-test</th>
</tr>
</thead>
<tbody>
<tr>
<td>35S::FT #11–1</td>
<td>Long day</td>
<td>3.1</td>
<td>0.4</td>
<td>3–4</td>
<td>7</td>
<td>a</td>
</tr>
<tr>
<td></td>
<td>Short day</td>
<td>3.1</td>
<td>0.3</td>
<td>3–4</td>
<td>10</td>
<td>a</td>
</tr>
<tr>
<td>35S::Hd3a-K #5–3</td>
<td>Long day</td>
<td>3.2</td>
<td>0.4</td>
<td>3–4</td>
<td>9</td>
<td>b</td>
</tr>
<tr>
<td></td>
<td>Short day</td>
<td>3.1</td>
<td>0.3</td>
<td>3–4</td>
<td>12</td>
<td>b</td>
</tr>
</tbody>
</table>

* P > 0.5
* P > 0.2
* Long day, 16-h light; short day, 8-h light.

Non-transgenic Columbia plants grown in similar conditions in a separate experiment had 15.8±2.3 rosette leaves (range 11–20) in long-day conditions and 53.8±2.1 rosette leaves (range 51–57) in short-day conditions.

![Fig. 3](image-url) Abundance of Hd3a mRNA under SD (A) and LD (B) conditions. RNAs were extracted from Nipponbare, NIL(Hd1), and NIL(Hd3a) grown under SD (10-h light) or LD (14-h light) conditions. Plants were sampled 1 h after the light was turned on. The vertical bars on each plot represent standard deviation. Days to heading in each line are given in parentheses.
Hd3a promotes flowering downstream of Hd1 in rice by quantitative RT-PCR. In Nipponbare, Hd1 was expressed throughout the light/dark cycle, with a peak at the beginning of the dark period under LD conditions. Under SD conditions, the levels of Hd1 mRNA were lower than under LD conditions, with a peak a few h after the beginning of the dark period (Fig. 5A). Hd3a also showed rhythmic expression under SD conditions (Fig. 5B), and was not detected under LD conditions (data not shown). In Nipponbare, Hd3a mRNA was abundant later in the dark period and in the middle of the light period. In NIL(Hd1), Hd3a mRNA was less abundant than in Nipponbare, although it seems to retain the rhythmic pattern under SD conditions.

**Discussion**

High-resolution mapping delimited a genomic region of about 20 kb as a candidate for Hd3a (Fig. 1). This region contains an open reading frame, cds25, which is similar to an Arabidopsis flowering-time gene, FT (Fig. 1D). Cds25 expression is strongly induced under SD conditions but not under LD conditions (Fig. 1E), which is consistent with SD-specific promotion of heading by Hd3a. Cds25 expression in NIL(Hd3a) was induced earlier and higher than in Nipponbare (Fig. 3). The introduction of Kasalath or Nipponbare alleles of cds25 into Nipponbare caused early heading in transgenic plants (Table 1, 2). In these experiments, the Kasalath allele tended to cause earlier transition to the reproductive phase than the Nipponbare allele. From these results, we concluded that cds25 is responsible for the Hd3a phenotype. This is the first report of the role of an FT-like gene in flowering in SD plants.

Hd3a shows about 70% identity with FT and 50% identity with TERMINAL FLOWER 1 (TFL1) (Fig. 1D). TFL1 shows sequence similarity with FT, but suppresses floral transition (Bradley et al. 1997, Ohshima et al. 1997). Overexpression of Hd3a and FT caused early heading in rice (Fig. 3A and S.K., Y.K., T.A. and M.Y., unpublished data) and early flowering in Arabidopsis (Table 3, 4). These facts suggest that Hd3a promotes the floral transition, as does FT. Sequence and functional similarities suggest that Hd3a is an ortholog of FT.

We found that several copies of FT-like genes exist in the...
**Materials and Methods**

**Plant materials**

F₁ plants were derived from a cross between a rice *japonica* cultivar, Nipponbare, and an *indica* cultivar, Kasalath. Backcrosses were carried out using Nipponbare as a recurrent parent. The population used for high-resolution mapping was self-pollinated progeny of a plant selected from the BC₄F₂ population in which the *Hd3a* region was heterogeneous and most other regions were homozygous for the Nipponbare allele. The NIL for *Hd3a* [NIL(*Hd3a*)] was described by Monna et al. (2002).

**Growth conditions of rice**

Rice plants were grown in a growth chamber at 26°C for 12 h and 22°C for 12 h, except during diurnal expression analysis, when they were grown at a constant 25°C. Experiments on overexpressing...
plants were done under SD (10.5-h light) or LD (13.5-h light) conditions with a photon irradiance of 300–350 μmol m⁻² s⁻¹.

Linkage analysis
We grew 2207 plants in a greenhouse. Recombinants around the Hd3a region were surveyed with CAPS markers as described by Monna et al. (2000). We used 18,432 clones in this library for PCR-based screenings with a photon irradiance of 300–350 μmol m⁻² s⁻¹

RT-PCR analysis
Total RNA was extracted from aerial parts of plants by using an SDS–phenol method. Total RNA (2 μg) was primed with dT₂₀ primer in a First-Strand cDNA Synthesis Kit (Amersham Biosciences) according to the manufacturer’s instructions. The cDNA was diluted to 20 μl with water, and 2 μl was used for each amplification. A rice actin gene (RAcI, GenBank accession number X16280) was used as a control. The amplification conditions were as follows: 2 min at 94°C; n cycles of 30 s at 94°C, 1 min at 60°C, and 1 min at 72°C; followed by 5 min at 72°C. The number of cycles is indicated in the captions to Fig. 1 and 5. Primer pairs were as follows: CP45 (5'-TCGAGACT-TCACACAGAAGG-3') and CP46 (5'-ACCTGTAATGTCGACATG-3') for Hd3a, OsACT1U (5'-TCACTTGGCCATCTCATCAG-3') and OsACT1L (5'-TGAGGACAAGCGATCAG-3') for actin, and MA2U (5'-GAGACGACGATGTTGA-3') and MA2L (5'-CATAGCCCTTCTTGTCCA-3') for Hdi1.

Real-time quantitative PCR
This was performed with TaqMan chemistry on an ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, U.S.A.) according to the manufacturer’s instructions. cDNA was synthesized as described above. Relative amounts were calculated as the ratio of the copy number of Hdi1 or Hdi3a to that of rice ubiquttin 2 (RUBQ2, GenBank accession number AF184280). Sequences for this experiment were as follows: Hdi1-1249F (5'-CGTGTGCCAGAAAGACAGGTA-3') and Hdi1-1314R (5'-AGATAGACGTGAGAGGAAC-3') for Hdi1 primers, Hdi1-1269F (5'-TGTCGAGTGAAAGACAGGTA-3') and Hdi3aF (5'-GACTGCTGGTCCATCAATTTCCA-3') for Hdi3a probe, Hdi3aR (5'-TGTCCGCACACATCGTAC-3') for Hdi3a Probe, CH262-497T (5'-ACCCCTTTCTTGTCCA-3') and CH262-543R (5'-ACTCGAGTGCATCAATAAAC-3') for RUBQ2 primers, and CH262-497T (5'-TGTCCGCACACATCGTAC-3') for RUBQ2 probe. All probes were 3'-labeled with TAMRA as a quencher dye. RUBQ2 probe was 5'-labeled with VIC. Other probes were 5'-labeled with FAM as a reporter dye.

Acknowledgments
We thank Drs. T. Baba and K. Yamamoto for sequence analysis of the PAC clone p0046E09 and Dr. T. Fuse for technical instruction in the transformation of rice. We also thank Dr. T. Izawa for critical reading of the manuscript. This work was supported by funds from the Program for Promotion of Basic Research Activities for Innovative Biosciences (PROBRAIN) and the Ministry of Agriculture, Forestry and Fisheries of Japan. The work in T.A.’s laboratory was supported by a Core Research for Evolutional Science and Technology (CREST) grant from the Japan Science and Technology Corporation and a Grant-in-Aid for Special Research on Priority Areas from the Ministry of Education, Culture, Sports, Science and Technology.

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(Received August 2, 2002; Accepted August 25, 2002)