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Rapid Paper

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

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***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

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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 *CK2*β caused early flowering in *Arabidopsis* (Sugano et al. 1999). These facts indicate that CK2 is involved in a photoperiod-dependent pathway in both species.

Hd3 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 covers 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 *Spe*I genomic fragment (Fig. 1C) carrying cds25 from Nipponbare or Kasalath into Nipponbare

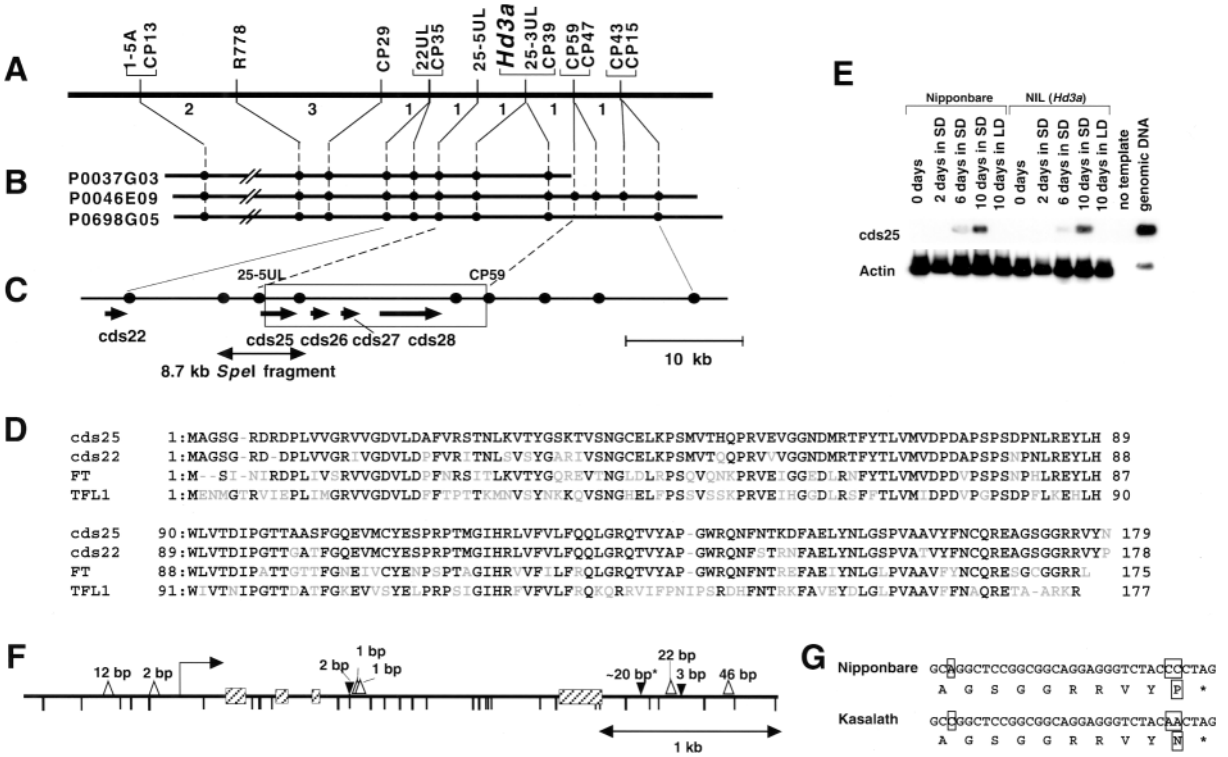

Fig. 1 Cds25 is a candidate gene for *Hd3a*. (A) Genetic linkage map showing the relative position of *Hd3a* on chromosome 6. The number of recombinant plants is indicated between markers. (B) Nipponbare PAC clones spanning the *Hd3a* region. (C) Detailed genetic and physical map of the *Hd3a* region. 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 *Spe*I 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 (T_0) 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 T_1 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_0 transgenic plants

		Days to heading [c] under SD conditions																		
		15	18	21	24	27	30	33	36	39	42	45	48	51	54	57	60	63	66	
Exp. 1, SD [a]	cds25K#1		1			1	1	1	3	2										
	cds25K#2	1			2	1		4												
	Vector									2	3	3	2	1						
Exp. 2, SD [a]	cds25N#1					1		2	1	1	3	1								
	Vector									2	5	1								
Exp. 3, SD [b]	cds25K#3				2	1	2	1		2	1	1	2	1						
	cds25N#2	1	1					1	1	3			1	1	1		1		1	
	Vector												1	2	2	2	2	1		

		Days to heading [c] under LD conditions																			
		15	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90	95	100	>100	N
Exp. 1, LD [b]	cds25K#1	1				1			1		2	1				1					5
	cds25K#2							1	1	1	2	2	2					1			1
	Vector																				6
Exp. 2, LD [a]	cds25N#1	2			1	2	1										1	1			2
	Vector															1	1	2	2	2	1

SD [a] and LD [a], plants were grown under SD (10-h light) and LD (13-h light) conditions with a photon irradiance of 600–700 μmol m^{-2} s^{-1}.
SD [b] and LD [b], 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^{-2} s^{-1}.
[c] Number of days required from transplanting to soil to the appearance of the first panicle.
cds25K#1-3, cds25N#1-2, and vector: plants transformed with pPZ-cds25K, pPZ-cds25N, and pPZ2H-lac vector (no insert). N, no panicle after 80 d from transplanting.

Table 2 Frequency distribution of days to heading in T_1 transgenic plants

		Days to heading under SD [a] conditions												
		33	36	39	42	45	48	51	54	57	60	63	66	69
Exp. 1	cds25K10-2					4	2	4	5 [b]	1 [b]				
	cds25K2-1				3	3	2		1 [b]					
	Nipponbare							1	4					
	NIL(*Hd3a*)					3	2							
Exp. 2	cds25K8-4	2	1	1	1	3						1 [b]		
	cds25N6-1								1	2	3			
	cds25N4-1									1	4/1 [b]	1/2 [b]		
	Nipponbare										2		2	1
	NIL(*Hd3a*)							3	2					

		Days to heading under LD [a] conditions																	
		35	40	45	50	55	60	65	70	75	80	85	90	95	100	105	110	115	>116	N
Exp. 1	cds25K10-2								1		5									2/ [b]
	cds25K2-1					1	1	1			1									5 [b]
	Nipponbare																			5
	NIL(*Hd3a*)																			5
Exp. 2	cds25K8-4	1		2	2	1	2													1 [b]
	cds25N6-1									1				1	1	1			2	1 [b]
	cds25N4-1																			7/2 [b]

SD [a] and LD [a], 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^{-2} s^{-1}. Under this LD conditions, neither Nipponbare nor NIL(*Hd3a*) headed at 130 d after sowing. cds25K10-2, cds25K2-1, and cds25K8-4: T_1 lines transformed with pPZ-cds25K. cds25N6-1, cds25N4-1, T_1 lines transformed with pPZ-cds25N. N, panicle did not appear within 130 d after sowing.
[b] Number of plants that did not inherit transgene.

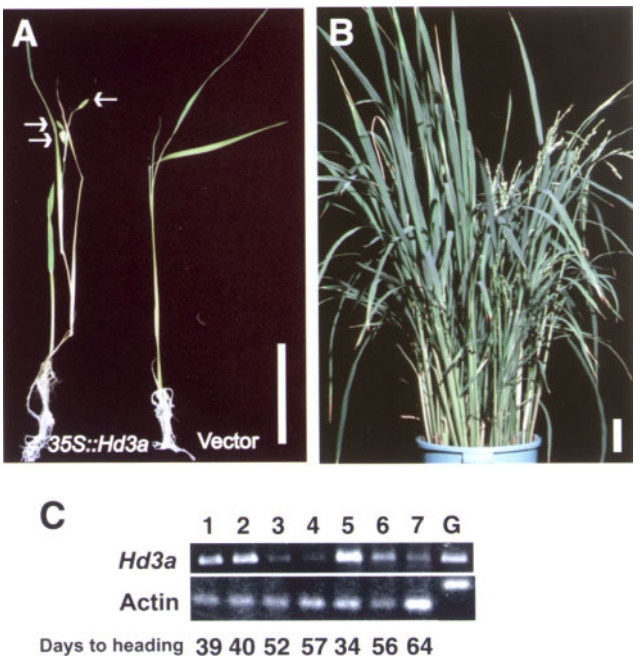

Fig. 2 Overexpression of sense and antisense *Hd3a* cDNA. (A) Effect of overexpression of *Hd3a*. Plant overexpressing *Hd3a* (left) and control plant carrying only selective marker (right). Arrows indicate spikelet-like structures. (B) Antisense expression of *Hd3a*. Plants with reduced level of *Hd3a* (left) showed late heading. Plants with usual level of *Hd3a* (right) had unaltered heading date. Bars indicate 100 mm. (C) RT-PCR analysis of *Hd3a* in the plants with antisense *Hd3a* (top). Actin was used as a control (bottom). G, genomic DNA control. For *Hd3a*, cDNA was synthesized by using CP46 primer to detect only sense *Hd3a* transcript. In this experiment, plants transformed with the empty vector headed at about 39 d after transplanting.

progeny of a plant with a single copy of the Nipponbare transgene, did not show such early heading. CdsN6-1, the progeny of a plant with two copies of the Nipponbare transgene, showed slightly early heading. Under LD conditions, some plants with the transgene headed, but not as early as under SD conditions, whereas plants without the transgene, Nipponbare, and NIL(*Hd3a*) had not headed by the end of the experimental period. Under LD conditions, we detected very low levels of *Hd3a* expression in control plants with the empty vector. In contrast, expression levels in plants with additional genomic copies were apparently higher than those of the control plants (data not shown). Accumulation of transcripts from more than two copies of *Hd3a* under LD conditions may result in threshold levels that promote floral transition. Another possibility is that unknown factors that repress *Hd3a* expression under LD conditions might be insufficient to suppress the expression of the introduced copies of the gene. These results indicate that cds25 of Nipponbare and Kasalath could promote heading, but the Kasalath allele is more effective than the Nipponbare allele.

From these results, we concluded that cds25 is responsible for the *Hd3a* phenotype. Supporting this conclusion, the 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 T_1 transgenic *Arabidopsis* overexpressing *FT* or *Hd3*

Transgene	Number of leaves	SD	Range	*N*
35S::FT	4.5	0.6	3–6	201
35S::Hd3a-K	4.1	0.6	2–6	223
35S::Hd3a-N	4.1	0.4	3–5	84
None [a]	10.7	0.9	8–12	54

[a] Non-transgenic Columbia plants grown on 1/2× MS – 1.5% sucrose media.

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 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 T_1 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*

Table 4 Photoperiodic response of transgenic *Arabidopsis* overexpressing *FT* or *Hd3a*

Transgenic line	Photoperiod [c]	Number of leaves	SD	Range	*n*	*t*-test
35S::FT #11–1	Long day	3.1	0.4	3–4	7	[a]
	Short day	3.1	0.3	3–4	10	[a]
35S::Hd3a-K #5–3	Long day	3.2	0.4	3–4	9	[b]
	Short day	3.1	0.3	3–4	12	[b]

[a] $P > 0.5$
[b] $P > 0.2$
[c] 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.

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 F_3 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 NIL(*Hd3a*) (Fig. 4A). Line 270-5 headed earlier than line 270-2. Corresponding to this early heading phenotype, levels of *Hd3a* mRNA in 270-5 were similar to those of NIL(*Hd3a*) and were higher than those in 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*

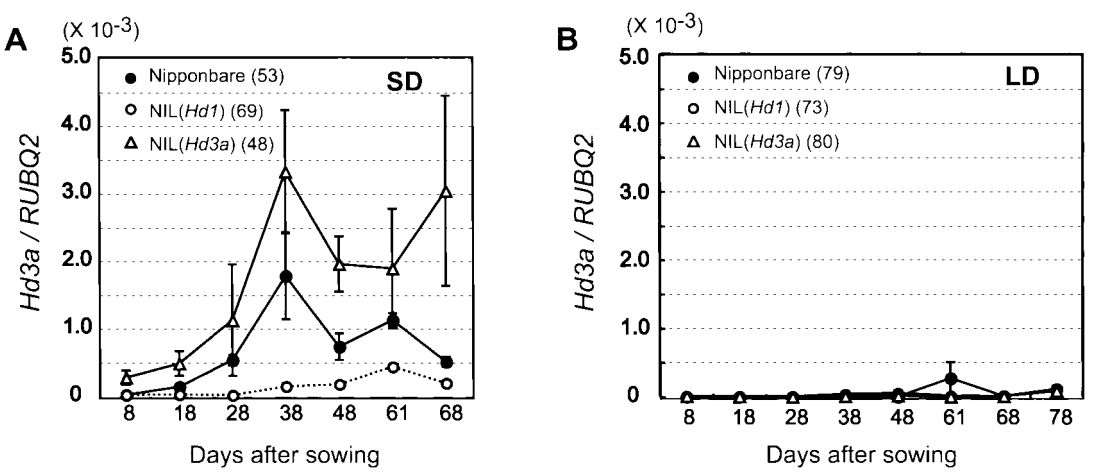

Fig. 3 Abundance of *Hd3a* mRNA under SD (A) and LD (B) conditions. RNAs were extracted from Nipponbare, NIL(*Hd1*), and NIL(*Hd3a*) grown under SD (10-h light) or LD (14-h light) conditions. Plants were sampled 1 h after the light was turned on. The vertical bars on each plot represent standard deviation. Days to heading in each line are given in parentheses.

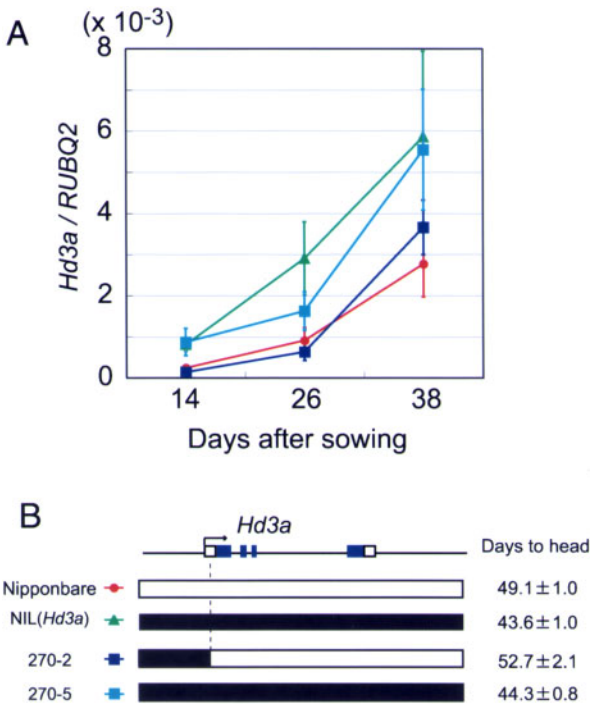

Fig. 4 (A) Abundance of *Hd3a* mRNA in homozygous progeny of recombinant in the *Hd3a* region. Nipponbare and NIL(*Hd3a*) are shown as controls. Plants were sampled 6 h after the light was turned on. The amount of *Hd3a* mRNA was higher than the results in Fig. 3A, since *Hd3a* expression is higher in the middle of day than in the morning (see Fig. 5). (B) Graphical genotype of *Hd3a* region in each line. Solid bars indicate the chromosomal segments of Kasalath introgressed into Nipponbare background (open bar). Average of days to heading and standard deviation of each line under SD conditions are shown on the right.

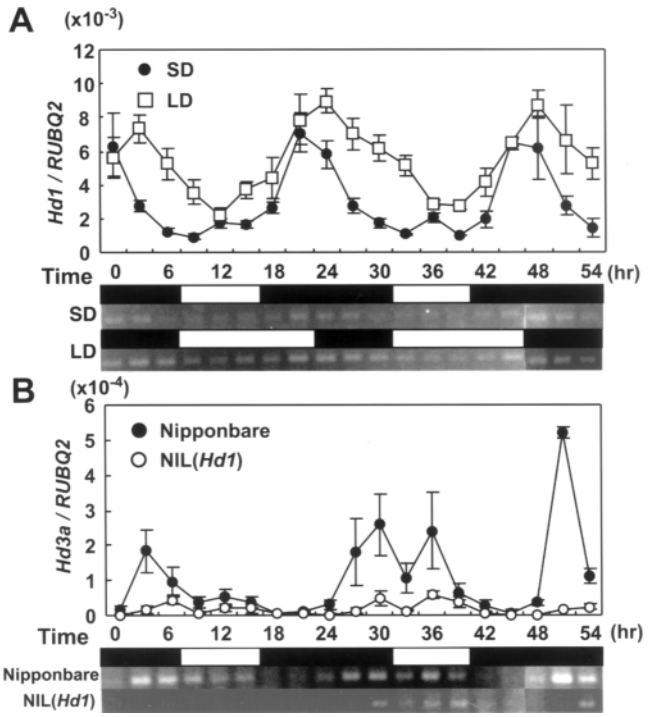

Fig. 5 Daily oscillation of *Hd1* and *Hd3a* abundance. (A) Relative amounts of *Hd1* mRNA in Nipponbare under SD and LD conditions. (B) Relative amounts of *Hd3a* mRNA in Nipponbare and NIL(*Hd1*) under SD conditions. Two or more independent experiments were performed; mean values are shown. The vertical bar on each plot represents standard deviation. Black and white bars indicate night and daytime. Ethidium bromide stainings of RT-PCR products amplified by using the same total RNA are shown below the graphs. The number of PCR cycles was 28 for *Hd1* and 35 for *Hd3a*. Plants were grown under LD conditions (15-h light) for 30 d and were subjected to an additional 7 d treatment under LD or SD conditions (9-h light) before sampling.

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

rice genome (data not shown). One of them, designated *RFT1*, lies adjacent to *Hd3a*, and its presumed amino acid sequence shows 91% identity with that of *Hd3a* (cds22 in Fig. 1D). *RFT1* also promoted heading when overexpressed (S.K. and M.Y., unpublished data). Under LD conditions, plants headed without any apparent increase in *Hd3a* expression (Fig. 3B). In natural field or LD conditions, other *FT*-like genes such as *RFT1* may have a role in promoting heading.

Both Kasalath and Nipponbare alleles of *Hd3a* are functional, because both promoted heading when genomic fragments were introduced into Nipponbare (Table 1, 2). We conclude that the Nipponbare allele was less functional than the Kasalath allele on the following criteria. First, the difference in heading dates between the two alleles was clear in an experiment using NILs (Monna et al. 2002). Second, the transgenic plants with the Kasalath allele tended to head earlier than those with the Nipponbare allele in T_0 and T_1 experiments (Table 1, 2). Nucleotide sequence variations were found between the two alleles: one results in an amino acid substitution and some may affect mRNA abundance. The amino acid substitution occurred at the carboxyl end of the predicted protein (Fig. 1G). The functional importance of the carboxyl terminal region was suggested from results in an *Arabidopsis ft-1* mutant (Kobayashi et al. 1999, Kardailsky et al. 1999). However, overexpression of both alleles in *Arabidopsis* resulted in the same degree of early-flowering phenotype (Table 3), whereas overexpression of *ft-1* cDNA did not (Y.K. and T.A., unpublished data), indicating that there is no significant allelic difference in the activity of the protein. On the other hand, *Hd3a* mRNA was detected earlier in NIL(*Hd3a*) than in Nipponbare under SD conditions (Fig. 3A, 4). It is likely that the difference in *Hd3a* mRNA levels and, possibly to a much lesser extent, a substitution at the carboxyl end are the cause of the allelic difference in phenotype. In this study, we could not clarify the nucleotide polymorphism that caused the allelic difference between Kasalath and Nipponbare *Hd3a*. We could exclude the possibility that the 5′ regulatory region conferred the difference in mRNA levels between Kasalath and Nipponbare *Hd3a* (Fig. 4). Several studies have reported that *cis*-elements in exon and intron regions generated differences in transcription levels (Douglas et al. 1991, Busch et al. 1999). It was also reported that a T-DNA insertion at 458 bp downstream of a putative polyadenylation signal in *TFL1* abolished the gene function in *tfl1-15*, suggesting the importance of the 3′ region (Ohshima et al. 1997). Thus, we consider that the nucleotide sequence downstream of the transcription initiation site of *Hd3a* might be important in determining *Hd3a* mRNA levels. Further analysis will be required to prove this hypothesis.

In NIL(*Hd1*), which has a non-functional *Hd1* allele, *Hd3a* expression was greatly reduced (Fig. 3, 5B). These results indicate that a functional Nipponbare *Hd1* enhances *Hd3a* expression under SD conditions. Consistent with this, overexpression of *Hd3a* in NIL(*Hd1*) caused early heading, as it did in Nipponbare (data not shown). Under LD conditions, little *Hd3a* transcript was detected, although *Hd1* expression was higher than under SD conditions. This indicates the importance of other factors in the regulation of *Hd3a* expression under LD conditions.

An emerging view from recent studies is that rice and *Arabidopsis* share multiple components that promote floral transition in response to opposite photoperiods (Yano et al. 2001). What causes this difference? One possibility is differences in the up-regulation of *Hd3a* and *FT*. We showed that *Hd3a*, a rice ortholog of *FT*, is induced under SD conditions and promotes heading. On the other hand, *Arabidopsis FT* is induced under LD conditions and promotes flowering (Kobayashi et al. 1999, Kardailsky et al. 1999). Therefore, *Hd3a* and *FT* share the same function, but they are induced by opposite photoperiod.

Up-regulation of *Hd3a* under SD conditions and *FT* under LD conditions requires *Hd1* and *CO*, respectively (this study and Kobayashi et al. 1999, Kardailsky et al. 1999, Samach et al. 2000). We showed that the expression of *Hd1* and *Hd3a* had distinct circadian patterns under SD and LD conditions (Fig. 5). High levels of expression of *Hd1* are restricted to the dark period under SD conditions, but were relatively high at dawn and dusk under LD conditions. Similar patterns in *CO* expression were also reported, although there were some differences (Suárez-López et al. 2001). In the case of *CO*, the importance of post-transcriptional regulation, possibly at the level of protein stability or translation efficiency, through interaction with light conditions has recently been suggested (Suárez-López et al. 2001). The abundance of *CO* mRNA was high at the beginning and end of the light period under inductive LD conditions in *Arabidopsis*. However, the abundance of *Hd1* mRNA was restricted to the dark period under inductive SD conditions in rice. These facts suggest that an important difference may be present in post-transcriptional regulation of Hd1 and CO, possibly through differential interaction with light conditions or in partner proteins with which Hd1 and CO proteins function. Elucidation of the molecular basis of this difference should deepen our understanding of the determination of photoperiod response in plants.

Materials and Methods

Plant materials

F_1 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 BC_3F_4 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. (2002). Progeny testing under SD conditions was performed as described by Monna et al. (2002). The nucleotide sequences of primers and restriction enzymes used to detect sequence polymorphism were as follows: CP13 (5′-GAGTAATTTGCGGTCAGAGTC-3′, 5′-CCAAACAACACATCCTCAG-3′ and *Tai*I), R778 (5′-CGTCGAGAAGCTCATAGCCA-3′, 5′-ATTTCACCGTCACACCACCC-3′ and *Xba*I), 22UL (5′-GTACCACTGGAGCAACATT-3′, 5′-ATACAGCTAGGCAGGTCTCA-3′ and *Tth*HB8I), CP17 (5′-GTTTCGTACATAAGACTGTT-3′, 5′-CCCTCACTTTTATTCATC-3′ and *Alu*I) CP29 (5′-AAATCGGGGACAGGTAATC-3′, 5′-TTCAAGTAACGAACGCCTAA-3′ and *Fnu*4HI), CP35 (5′-TACGGGCTACGGACATAACA-3′, 5′-CGCTCAGCAACGAGTTTC-3′ and *Tth*HB8I), 25-5UL (5′-ATGGGAGGCTATATCAACTG-3′, 5′-TCCAGCACATCACCCACAA-3′ and *Hae*III), 25-3UL (5′-TCAGAACTTCAACACCAAGG-3′, 5′-ACCTTAGCCTTGCTCAGCTA-3′ and *Hae*III), CP39 (5′-GGGAGAATATGTTGCAGTAG-3′, 5′-CAAATGGTAATGGGTCAA-3′ and *Alu*I), CP59 (5′-AGCCTCTGCGTCACTGTCATC-3′, 5′-GCAGCAGCAAACTCCCAAAG-3′ and *Tth*HB8I), CP47 (5′-GACTACCCGCTGTACCAC-3′, 5′-ATGACATCTCACCATTTCAC-3′ and *Tsp*EI) and CP43 (5′-AGTGTCATTGTCCCGCTAT-3′, 5′-TTCACAAACAAGGGGTAAAG-3′ and *Ssp*I).

Screening of genomic clone of Hd3a region

A PAC library with the Nipponbare genome was used (Baba et al. 2000). We used 18 432 clones in this library for PCR-based screening with DNA markers CP13, R778, and CP39. A Kasalath genomic library was constructed with the binary cosmid vector pPZP2CH-lac (Fuse et al. 2001). The library was screened with two amplified fragments from CP35 and CP39 as hybridization probes by using the ECL direct-labeling and detection system (Amersham Biosciences, Piscataway, NJ, U.S.A.).

Transgenic analysis in rice

For complementation tests, the 8.7-kb *Spe*I genomic fragments of Kasalath and Nipponbare containing cds25 (Fig. 1C) were subcloned into binary vector pPZP2H-lac (Fuse et al. 2001) to create pPZ-cds25K and pPZ-cds25N, respectively. For overexpression experiments, cDNAs were synthesized from SD-treated NIL(*Hd3a*). The *Hd3a* fragment was amplified by using flanking primers 5′-GCTGCCTCTATCACAGTATATT-3′ (sense) and 5′-ACCTTAGCCTTGCTCAGCTA-3′ (antisense), and cloned into pPZP2Ha3 (Fuse et al. 2001) downstream of the 35S cauliflower mosaic virus promoter. We introduced them into Nipponbare by *Agrobacterium*-mediated transformation (Fuse et al. 2001). Regenerated shoots were incubated for several days under continuous light to induce roots. Then they were transferred to soil and were grown under SD or LD conditions.

Transgenic analysis in Arabidopsis

Plants of ecotype Columbia were transformed with the same constructs used in rice or with *FT* cDNA cloned into the same vector by vacuum infiltration (Bechtold et al. 1993). Self-pollinated seeds (T_1) of vacuum-infiltrated plants were sown on 1/2 MS – 1.5% sucrose medium supplemented with hygromycin (10 mg $liter^{-1}$) for selection of transgenic seedlings. Transgenic plants were grown in LD conditions (16-h light, 75 μmol m^{-2} s^{-1}) at 22°C, and the total number of leaves (rosette and cauline) was counted after bolting. A homozygous line of plants overexpressing *Hd3a* from Kasalath (*35S::Hd3a-K* #5-3) was selected and examined for photoperiod response. Lines *35S::Hd3a-K* #5-3 and *35S::FT* #11-1 (Kobayashi et al. 1999) were grown on soil in LD (16-h light, 135 μmol m^{-2} s^{-1}) or SD (8-h light, 200 μmol m^{-2} s^{-1}) conditions at 22°C, and the total number of leaves was counted after bolting.

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 dT_{18} 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 (*RAc1*, 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 follow: CP45 (5′-TCAGAACTTCAACACCAAGG-3′) and CP46 (5′-ACCTTAGCCTTGCTCAGCTA-3′) for *Hd3a*, OsACT1U (5′-TCCATCTTGGCATCTCTCAG-3′) and OsACT1L (5′-GTACCCGCATCAGGCATCTG-3′) for actin, and MA2U (5′-GAGCAGCAGCATAGTGGTTA-3′) and MA2L (5′-CATACGCCTTTCTTGTTTCA-3′) for *Hd1*.

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 *Hd1* or *Hd3a* to that of rice *ubiquitin 2* (*RUBQ2*, GenBank accession number AF184280). Sequences for this experiment were as follow: Hd1-1249F (5′-CGTTTCGCCAAGAGATCAG-3′) and Hd1-1314R (5′-AGATAGAGCTGCAGTGGAGAAC-3′) for *Hd1* primers, Hd1-1269T (5′-TGTGCAGATCGAAGTGGACCAGA-3′) for *Hd1* probe, Hd3aF (5′-GCTAACGATGATCCCGAT-3′) and Hd3aR (5′-CCTGCAATGTATAGCATGC-3′) for *Hd3a* primers, Hd3aP (5′-CTGCTGCATGCTCACTATCATCATCC-3v) for *Hd3a* probe, CH262-476F (5′-GAGCCTCTGTTCGTCAAGTA-3′) and CH262-543R (5′-ACTCGATGGTCCATTAAACC-3′) for *RUBQ2* primers, and CH262-497T (5′-TTGTGGTGCTGATGTCTACTTGTGTC-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.

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