

Hd1*, a Major Photoperiod Sensitivity Quantitative Trait Locus in Rice, Is Closely Related to the Arabidopsis Flowering Time Gene *CONSTANS

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A major quantitative trait locus (QTL) controlling response to photoperiod, *Hd1*, was identified by means of a map-based cloning strategy. High-resolution mapping using 1505 segregants enabled us to define a genomic region of ~12 kb as a candidate for *Hd1*. Further analysis revealed that the *Hd1* QTL corresponds to a gene that is a homolog of *CONSTANS* in Arabidopsis. Sequencing analysis revealed a 43-bp deletion in the first exon of the *photoperiod sensitivity 1* (*se1*) mutant HS66 and a 433-bp insertion in the intron in mutant HS110. *Se1* is allelic to the *Hd1* QTL, as determined by analysis of two *se1* mutants, HS66 and HS110. Genetic complementation analysis proved the function of the candidate gene. The amount of *Hd1* mRNA was not greatly affected by a change in length of the photoperiod. We suggest that *Hd1* functions in the promotion of heading under short-day conditions and in inhibition under long-day conditions.

INTRODUCTION

The transition of the apical meristem from vegetative to reproductive growth is a critical event in the life cycle of a plant. In rice, the timing of this transition affects the timing of heading. This timing, or heading date, is one of the critical traits considered for adapting rice to different cultivation areas and cropping seasons. Rice is a short-day (SD) plant; its heading is promoted by short photoperiods. The response of the plant to length of day (referred to as photoperiod sensitivity [PS]) and its basic vegetative growth determine the heading date of rice. Many genetic studies of heading date have been performed, and several genes controlling PS in rice have been genetically identified, including *Se1* (*Lm*), *Se3* to *Se7*, and *E1* to *E3* (Yokoo et al., 1980; Yamagata et al., 1986; Poonyarit et al., 1989; Sano, 1992; Yokoo and Okuno, 1993; Tsai, 1995; Kinoshita, 1998). However, only one gene involving photoperiod response in rice has been cloned, *Se5* (Izawa et al., 2000). Little is known about the structure and function of PS genes in rice at the molecular level. In contrast, several genes involved in flowering time

have been isolated, allowing clarification of part of the genetic control pathway in Arabidopsis (reviewed by Levy and Dean, 1998; Fowler et al., 1999; Kobayashi et al., 1999; Sheldon et al., 1999). Identification of the genes involved in flowering time has made it possible to determine the genetic control pathways for the response to photoperiod and vernalization in Arabidopsis (reviewed by Levy and Dean, 1998; Samach and Coupland, 2000). In addition, homologs of Arabidopsis genes for flowering time also function in *Brassica napus* (Robert et al., 1998).

The major genes or quantitative trait loci (QTLs) for heading date have been mapped by using DNA markers in rice (Mackill et al., 1993; Li et al., 1995; Xiao et al., 1996; Yano et al., 1997; Lin et al., 1998; Tamura et al., 1998). Four QTLs for heading date involved in PS were mapped precisely as single Mendelian factors by using advanced backcross progeny (Yamamoto et al., 1998, 2000; Lin et al., 2000). A major gene controlling PS, *Se1*, first was identified as a naturally occurring variant (Yokoo et al., 1980) and was thought to lie at the same locus as the PS QTL *Hd1*, based on a comparison of their map locations (Yano et al., 1997; Tamura et al., 1998; Yamamoto et al., 1998). Mutant lines with less PS were induced, and some were caused by mutations in the *Se1* locus (Inoue et al., 1992). However, no direct evidence was available to prove their allelic relationship, and isolation

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of the QTL *Hd1* or *Se1* gene might be an effective way to prove allelism. Linkage mapping for *Se1* with restriction fragment length polymorphism (RFLP) markers has been conducted, but the resolution was insufficient for map-based cloning of *Se1*. To analyze the genetic control of rice heading at the molecular level, we attempted to isolate the genes involved by using naturally occurring variations and a quantitative genetic approach (Yano and Sasaki, 1997). One QTL for PS, *Hd6*, identified in a recent study, has been isolated by map-based cloning (Y. Takahashi and M. Yano, unpublished data).

In this article, we report the isolation of a major rice PS QTL, *Hd1*, by map-based cloning. We found that *Hd1* is a homolog of *CONSTANS* from Arabidopsis and encodes a protein with a zinc finger domain. We also confirmed by structural and expression analysis that the major gene controlling PS, *Se1*, is allelic to *Hd1*. The nucleotide sequence data reported in this article have been deposited in the DNA Data Bank of Japan nucleotide sequence databases with the accession numbers AB041837 (Nipponbare *Hd1*; genomic), AB041838 (Nipponbare *Hd1*; cDNA), AB041839 (Kasalath *Hd1*), AB041840 (Ginbouzu *Hd1*), AB041841 (HS66 *Hd1*), and AB041842 (HS110 *Hd1*).

RESULTS

Fine-Scale and High-Resolution Mapping

Of >9000 BC₃F₃ plants grown under natural field conditions, nearly 2000 exhibited early heading, indicating that those plants were homozygous for the recessive Kasalath allele at the *Hd1* locus. Yamamoto et al. (1998) reported that early heading plants were homozygous for the Kasalath allele at *Hd1*, in contrast with plants that were homozygous or heterozygous for the Nipponbare allele, which were later heading under natural field conditions. To avoid contamination by plants heterozygous at *Hd1*, we carefully selected 1505 plants that showed extreme early heading. Two RFLP markers that flank *Hd1*, R1679 and P130 (Yamamoto et al., 1998), were used to detect recombination events between *Hd1* and markers in the pooled DNAs. Nine and two recombinant chromosomes were detected among 301 pools for R1679 and P130, respectively (Figure 1). Plants containing the recombinant chromosome in each pool were identified from their progeny. The total DNA of these selected plants was used to develop a fine-scale map of the *Hd1* region. Eleven plants were selected and analyzed with additional RFLP markers (C235 and S2539) and a cleaved amplified polymorphic sequence (CAPS) marker (S20481) that had been developed from random expressed sequence tag (EST) clones (Yamamoto and Sasaki, 1997). The results suggest that *Hd1* lies in the interval between S20481 and P130 (Figure 1).

Yeast artificial chromosome clones containing markers flanking *Hd1* have been screened in the physical mapping project of the Rice Genome Research Program (RGP; Kurata et al., 1997). Two yeast artificial chromosome clones, Y4836 and Y3955, were found to contain three flanking markers, C235, S20481, and S2539. The end DNA sequence of Y4836 was cloned; RFLP analysis showed that the resulting clone, Y4836R, cosegregated with P130 (Figure 1). Moreover, two P1-derived artificial chromosome (PAC) clones, P0676F10 and P0038C5, were selected with the markers S20481, S2539, and Y4836R from the PAC genomic library of Nipponbare developed at RGP (Baba et al., 2000). P0038C5 contained sequences for S20481, S2539, and Y4836R. This suggested that P0038C5 encompassed the *Hd1* locus (Figure 1).

P0038C5 was sequenced by means of a shotgun strategy. A region of ~26 kb was defined as an *Hd1* candidate region, based on the position of the flanking markers S20481 and Y4836R. To determine the location of *Hd1* more precisely, we developed nine CAPS markers (1a to 1i), by using sequence data within the 26-kb candidate genomic region. These CAPS markers detected recombination events between markers 1a and 1b, 1e and 1f, and 1i and Y4836R; four markers—1b, 1c, 1d, and 1e—cosegregated with *Hd1* (Figure 1). As a result, a sequence of ~12 kb was defined as the *Hd1*-containing region.

Identification and Analysis of the *Hd1* Sequence

The candidate genomic sequence was analyzed with the Genscan program (<http://genes.mit.edu/GENSCAN.html>). Two putative genes were predicted in this region (data not shown). A BLAST search of nonredundant DNA databases revealed that one putative gene showed considerable similarity to the Arabidopsis *CONSTANS* (*CO*) gene. Another probable candidate was a sequence identical to that for peroxidase S2539, which was found in random EST sequencing at RGP. At this stage, there was no evidence to exclude S2539 as a candidate for *Hd1*. However, the sequence showing similarity to *CO* was further analyzed as a candidate because of the known function of *CO* in photoperiod response in Arabidopsis (Putterill et al., 1995).

A cosmid clone containing the candidate genomic region was obtained by screening a genomic library of Kasalath. We determined the sequence for a candidate 12-kb region of Kasalath. Comparison of the sequences of Nipponbare and Kasalath revealed many sequence variations. We found four single-base substitutions, one two-base substitution, a 36-bp insertion, and a 33-bp deletion in the putative first exon as well as two single-base substitutions and a two-base deletion in the putative second exon (Figure 2).

Using several kinds of primer pairs that could amplify genomic sequences of the *Hd1* candidate, we analyzed the sequences of the *se1* mutants HS66 and HS110 and their progenitor variety, Ginbouzu. On the basis of sequence

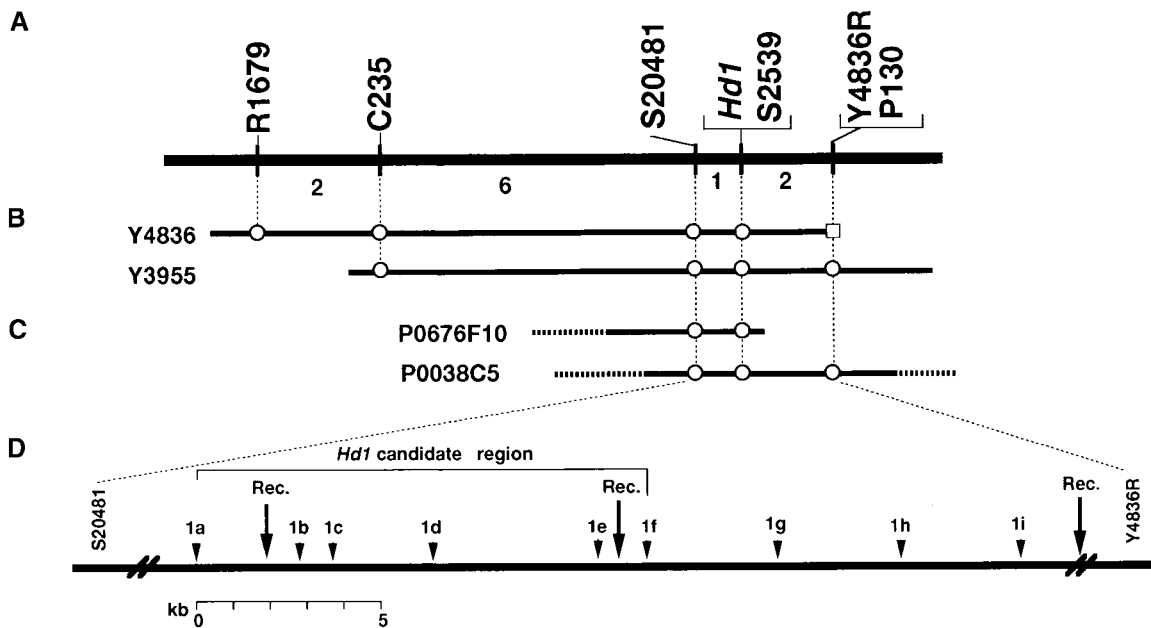


Figure 1. A Fine-Scale, High-Resolution Genetic and Physical Map of the *Hd1* Region on Chromosome 6.

(A) Genetic linkage map showing the relative position of *Hd1* with RFLP markers on chromosome 6. Numbers under the horizontal line are numbers of plants with a recombinant chromosome in the adjacent marker intervals.

(B) and **(C)** Yeast artificial chromosome **(B)** and PAC clones **(C)** spanning the *Hd1* region. A circle indicates the existence of a sequence corresponding to the RFLP markers. Entire insert sequencing was performed on PAC clone P0038C5.

(D) Detailed genetic and physical map showing the relative positions of the candidate regions of *Hd1* and CAPS markers developed based on sequence data. Rec., approximate positions of recombination events that occurred near *Hd1*.

comparison with the Ginbouzu *Hd1*, we found a 43-bp deletion in the putative first exon of *se1* mutant HS66 and a 433-bp insertion in the putative intron of HS110. These results clearly suggested that *Hd1* was allelic with *Se1*. Moreover, we found one single-base substitution and a 36-bp insertion in the first exon of the Ginbouzu *Hd1* sequence, compared with that of Nipponbare *Hd1* (Figure 2), although both alleles were functional for photoperiod response. The insertion was located at the terminal region of the zinc finger domain (Figures 2A and 3B). Although many variations occurred between the *Hd1* sequences of Nipponbare and Kasalath outside of the coding region as well as in the coding regions, no difference was found between Nipponbare and Ginbouzu outside of the coding region. Both Nipponbare and Ginbouzu belong to the *japonica* subgroup of *Oryza sativa*, which shows very small genetic variation.

In addition to genomic sequencing of *Hd1*, using the rapid amplification of cDNA ends method, we determined the cDNA sequence of *Hd1* by sequencing a product amplified by reverse transcription-polymerase chain reaction (RT-PCR) and 3' and 5' primer extension. We thus verified the putative exons and intron based on a prediction from the Genscan maize model. When we compared the sequence of

Hd1 with that of *CO* from Arabidopsis, we found 59% identity in the zinc finger domain and 79% identity in the C-terminal region. The sequence obtained indicates that rice *Hd1* is composed of two exons that encode a 395-amino acid protein and is a member of the Arabidopsis *CO* family with a zinc finger domain (Figure 3A). The deduced amino acid sequence of the *Hd1* protein was compared with that of *CO* from Arabidopsis and *BnCOA1* from *B. napus*. Comparison of two domains conserved in most members of the *CO* family revealed substantial similarity among them (Figure 3A). The region containing the zinc finger motif showed 65% identity and a consensus structure of $CX_2CX_{16}CX_2C$ in the *CO* family (Putterill et al., 1995; Robert et al., 1998). The region near the C terminus showed 83% identity and is thought to be a nuclear localization signal (Putterill et al., 1995; Robert et al., 1998). Downstream from the zinc finger, the Nipponbare *Hd1* sequence had little similarity with the sequences of the Arabidopsis *CO* and *B. napus* *BnCOA1* proteins (Figure 3A). The basic amino acid motif (RRHQR) that is a common feature between the Arabidopsis and *B. napus* *CO* family (Robert et al., 1998) in the C terminus of the zinc finger domain was present in the Ginbouzu *Hd1* protein but not in Nipponbare (Figure 3B).

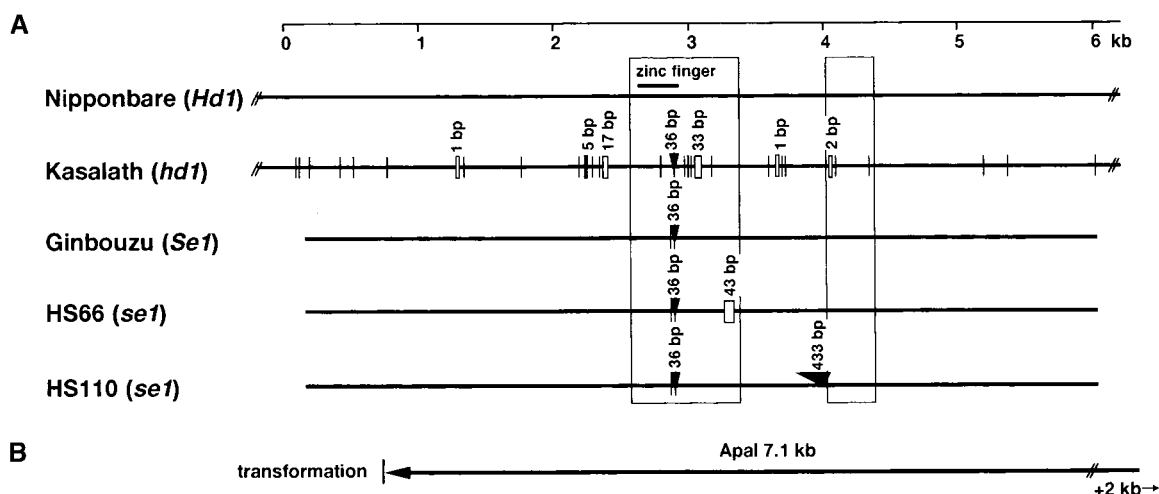


Figure 2. Scheme of the Structural Differences in the Candidate Region of *Hd1* in Nipponbare and Kasalath and the Corresponding Genomic Region of *se1* Mutants HS66 and HS110 and Their Original Variety, Ginbouzu.

(A) Comparison of genomic sequences of Nipponbare and Kasalath *Hd1* alleles. Boxes show the predicted open reading frames based on the Genscan software maize model. Vertical lines without labels represent single-base substitutions between Nipponbare and Kasalath. Small rectangular boxes and arrowheads represent deletions and insertions, respectively.

(B) A 7.1-kb Apal genomic fragment containing the entire *Hd1* candidate sequence used in the complementation analysis. This fragment does not contain another predicted gene, encoding peroxidase, which was found in the candidate genomic region of *Hd1*.

Functional Complementation with Candidate Gene in Transgenic Rice

A 7.1-kb Apal fragment of Nipponbare (Figure 2B) containing the candidate *Hd1* region was transferred into a nearly isogenic line (NIL) of Nipponbare, NIL(*Hd1/Hd2*). The recipient line exhibited no photoperiod response, because Nipponbare functional alleles of *Hd1* and *Hd2* were replaced with Kasalath nonfunctional alleles (Lin et al., 2000). Thus, the transgenic plants with the Nipponbare *Hd1* sequence were expected to exhibit a more sensitive photoperiod response, such as a promotion of heading in SD conditions. Fifty plants with a hygromycin resistance gene and containing the target gene region were obtained by Agrobacterium-mediated transformation with a 7.1-kb Apal fragment (Figure 2). The Apal fragment contains only one gene highly similar to Arabidopsis *CO*. Some transgenic plants showed earlier heading in a growth chamber under SD conditions than did those with only the vector sequence and the nearly isogenic line NIL(*Hd1/Hd2*) control (data not shown). We selected one transgenic plant that showed early heading and had one copy of the 7.1-kb fragment for further progeny analysis. Self-pollinated progeny of the selected plant showed wide variation in days to heading (53 to 93 days) under SD conditions (Figure 4). We used CAPS marker 1e to determine the presence or absence of the gene in each plant. Plants without the transgene and NIL(*Hd1/Hd2*) plants showed later heading than did plants homozygous or heterozygous for the transgene. All transgenic plants showed

no growth abnormalities. Thus, the 7.1-kb candidate genomic region promoted heading under SD conditions. This is consistent with results comparing NIL(*Hd2*) and NIL(*Hd1/Hd2*) plants (Lin et al., 2000). These results clearly suggest that the *Hd1* sequence in the 7.1-kb candidate genomic region retains the function of photoperiod response.

Expression of *Hd1*

We could not detect *Hd1* mRNA in RNA gel blot analysis. Thus, to determine whether the *Hd1* candidate region was expressed and whether the expression of *Hd1* was induced by a change of daylength, we performed RT-PCR analysis (Figure 5). The amplified fragment was designed to include a 33-bp deletion in the first exon in Kasalath and a 43-bp deletion in the first exon in HS66 but not a 36-bp insertion in the first exon in Kasalath or Ginbouzu (Figure 2). The fragment also included a 433-bp insertion in the intron in HS110. *Hd1* mRNA was detected in Nipponbare, Kasalath, and NIL(*Hd1*). However, the amplified product was slightly smaller in Kasalath than in Nipponbare. Sequencing the amplified product showed this size difference to be consistent with the 33-bp difference in the genomic sequences (Figure 2). Ginbouzu produced the same amount of mRNA as Nipponbare. The *se1* mutant HS66 also produced the same amount of mRNA as Ginbouzu, but its PCR product was slightly smaller than that of Ginbouzu. On the other hand, several different amplified products of the *se1* mutant

HS110 were seen in the RT-PCR assay (Figure 5). Sequencing these RT-PCR products revealed a 43-bp deletion in the amplified product of HS66 and a 433-bp insertion in the amplified product of HS110. A sequencing product the same as that of Nipponbare also was seen in HS110 (data not shown). These changes are consistent with the results of genomic sequencing of HS66 and HS110 (Figure 2). Thus, the transcription of *Hd1* may have been inaccurate in the *se1* mutant HS110.

The quantities of Nipponbare mRNA at *Hd1* did not change with the transition from long-day (LD) to SD conditions (Figure 5), which was associated with the initiation of transition to heading. We also observed no differences in *Hd1* mRNA amounts when the plants were grown solely in SD or LD conditions (data not shown). These results suggest that the expression of *Hd1* is not greatly affected by a change of length of photoperiod.

DISCUSSION

Map-Based Cloning of QTLs

Many genes for heading in rice have been genetically identified (Yokoo et al., 1980; Yamagata et al., 1986; Poonyarit et al., 1989; Yokoo and Okuno, 1993; Tsai, 1995; Kinoshita, 1998). Several were suggested to involve response to photoperiod (i.e., PS). However, little is known about the genetic control pathway of PS, owing to a lack of molecular information on the genes involved. In this study, we cloned *Hd1*, one of the genes that control PS in rice, by map-based cloning with naturally occurring allelic variations. Another QTL, *Hd6*, which also is involved in photoperiod response, has been isolated by the same map-based strategy (Y. Takahashi and M. Yano, unpublished data). These results imply that QTLs detected based on naturally occurring allelic variation can be isolated with the use of advanced backcross progeny (Yano and Sasaki, 1997; Yamamoto et al., 1998, 2000). In rice as in Arabidopsis (Alonso-Blanco and Koornneef, 2000), naturally occurring variation can provide new gene sources for plant genetics and molecular biology.

Similarity in Structure of *Hd1* and CO

The *Hd1* protein contains two zinc finger domains of structure CX₂CX₁₆CX₂C. The zinc finger domain is believed to be involved in DNA binding in the GATA1 gene family (Pevny et al., 1991; Omichinski et al., 1993). The C-terminal region of *Hd1*, which is thought to be a nuclear localization signal, also showed considerable identity with the CO family at the amino acid level (Robert et al., 1998). Several ESTs in rice exhibited a conserved structure with the CO family (Putterill et al., 1995; Song et al., 1998), and three of them showed

strong similarity at the amino acid level to CO in the N-terminal and C-terminal regions. However, *Hd1* is more closely related to CO than to proteins encoded by the three rice ESTs. Linkage mapping of these ESTs revealed no candidate ESTs for *Hd1* (Song et al., 1998), perhaps because of the very low expression of *Hd1* as revealed in this study. We have shown here that *Hd1*, a CO homolog in rice, also has a role in photoperiod response.

Allelic Relationship between *Hd1* and *Se1* and Loss of Function in Kasalath *Hd1* and *se1* Mutant Alleles

Genetic analyses of PS in several Japanese rice varieties (Okumoto et al., 1991; Ichitani et al., 1998) indicate that Nipponbare might possess an allele of *Se1* with a strong photoperiod response. Genetic linkage mapping revealed that the chromosomal location of *Hd1* coincided with that of *Se1* (Tamura et al., 1998; Yamamoto et al., 1998), but no direct evidence demonstrated an allelic relationship between *Hd1* and *Se1*. In this study, we identified the allelic relationship by structural and expressional analysis of *Hd1*.

The Nipponbare and Kasalath *Hd1* alleles have many structural differences between them (Figure 2). A 2-bp deletion in the second putative exon in the Kasalath allele results in a premature stop codon. Thus, the Kasalath *Hd1* protein could be shorter than the Nipponbare protein because it is missing the C-terminal region (Figure 2). The 43-bp deletion in the *se1* mutant HS66 also produces a premature stop codon. Abnormal transcripts with a 433-bp insertion sequence in the intron region were detected in the *se1* mutant HS110 by RT-PCR. However, small amounts of mRNA of the same size as in Nipponbare also were detected in this mutant (Figure 5), and the sequence of this product is identical to that of Nipponbare. Days to heading of HS110 was ~4 days later than that of HS66 under natural field conditions (data not shown). This finding indicates that the function of the *se1* allele in HS110 was not completely lost (data not shown). This phenotypic difference might reflect the presence of normal-size transcripts in HS110. Perhaps HS110 might be categorized as a leaky mutant at the *Hd1* locus.

We also found a structural difference between the functional *Hd1* alleles of Nipponbare and Ginbouzu. A 36-bp nucleotide sequence corresponding to the terminal region of a zinc finger domain and including the basic amino acid motif RRRQR was present in the Ginbouzu *Hd1* allele but not in the Nipponbare allele (Figures 2A and 3B). This sequence was also present in Kasalath, which makes it more likely that a 36-bp deletion occurred in the Nipponbare *Hd1* allele. This region is thought to be important for DNA–DNA interaction (Omichinski et al., 1993) and is highly conserved between rice and Arabidopsis or *B. napus* (Robert et al., 1998). At present, no concrete evidence explains the functional difference between the Nipponbare and Ginbouzu *Hd1* alleles. Clarifying any functional difference between them by genetic

A

Hd1	MNYNF GGNVFDQ EVGVGGEGGGGGEGSGCPW ARPCDGCRAAPSVVYCRADAAAYLCASCDARVHAANRVASRRH RV	75
CO	M-----L KQE ---SNDIGSGENNR----- ARPCDTCRSNACTVYCHADSAYLCMSCDAQVHSANRVASRRHKRV	60
BnCOA1	M-----F KQE ---SNNIGSEENNTG--- FRACDTCGSTICTVYCHADSAYLCNSCDAQVHSANRVASRRHKRV	61
Consensus	M.....QE.....G.....R.CD.C.....VYC.AD.AYLC.SCDA.VH.ANRVASRRH.RV	
▼▼▼▼		
Hd1	RVCEACERAPAAALACRADAALCVACDVQVHSANPL-----PAITIPATSVLAEAVVATATVLGDKDE	138
CO	RVCESCERAPAAFLCEADDA SLCTACDSEVHSANPLARRHQRVPL LPISGNSFSSMTTTHHQSEKMTDPEKRLV	135
BnCOA1	RVCESCERAPAAFMCEAD VDVSLCTACDLEVHSANPLARRHQRVPL VVPITGNSCSSLATANH---TTVTEPEKRVV	133
Consensus	RVCE.CERAPAA..C.AD...LC.ACD..VHSANPL.....T.....	
Hd1	EVDSWLLLSKSDSDNNNNNNNDNDNDNNNSNSNNGMYFGEVDEYFDLVGYNSYYDNRIENNQDRQYGMHEQQ	213
CO	VDQEEGEGDKDAKEVASWLF PNS ---DKNNNNQNNGLLFS--DEYLNLDVYNSSMDYKFTGEYSQHQQN---C	201
BnCOA1	LVQE-----DAKETASWLF PKNSDNH ---NNNNONNELLS--DDYLDLADYNSMDYKFTGQYNQPTQHKQDC	197
ConsensusN.N.NN...F...D.Y..L..YNS..D.....	
Hd1	EQQQQQQEMQKEFAEKEGSEC VVPSQITMLSEQHSGYGVGADQAASMTAGVSAITD SISNSISFSSMEAGIVP	288
CO	SVPQTSYGGDR-----V VPLKLEESRGRHQCHNQNFQFN IKYSSGTHYNDNGSINHNAIYISSMETGVVVP	266
BnCOA1	TVPEKNYGGDR-----V VPLQLEETRGNLHHKQH NIT---YSSGSHYNNNGSINHNAIYNP SMETDFVP	258
ConsensusVVP.....SI.....SME...VP	
Hd1	DSTVIDMPNSRILTPAGAINLFSG P SLOMS--LHSSMDREARV LYREK KKAR K FEKTIRY ET RKAYAEAR P RI	361
CO	ESTACVTTASHPRTPKGTVEQ QDPASQMITVTQ SPMDREARV LYREK RRK R FEKTIRYAS R KAYAEIR P RV	341
BnCOA1	EQTAPDKTVSHPKTHGKIEK LPEPLIQI -----I SPMDREARV LYREK KKRR KFEKTIRYAS R KAYAE R RPRI	328
Consensus	..T.....S...T..G.....P..Q.....S.MDREARVLYREK.K.RKFEKTIRY..RKAYAE.RPR.	
Hd1	<u>KGRFAK</u> RS SDVQIEVDQMFSTAALSDGSYG TVPWF	395
CO	<u>NGRFAK</u> -REIEAEEQGFNTMLM-YNTGYGIVPSF	373
BnCOA1	<u>NGRFAK</u> IS ETEVEDQ EYNTMLMYDTGYGIVPSFYGQK	366
Consensus	.GRFAK.....E.....YG.VP.F....	

B

Nipponbare <i>Hd1</i>	98 CVACDVQVHSANPL-----PAITIPATSVLAEAV	126
Ginbouzu <i>Hd1 (Se1)</i>	98 CVACDVQV YSANPLARRHQRV PA PL PAITIPATSVLAEAV	138

Figure 3. Deduced Amino Acid Sequence of Hd1 Protein, Amino Acid Alignment with Arabidopsis CO and *B. napus* BnCOA1, and Comparison of Amino Acid Sequences of C-Terminal Regions of the Zinc Finger Domain between Nipponbare and Ginbouzu Hd1.

(A) Comparison of Nipponbare Hd1, CO, and BnCOA1 alleles. Boxes in N and C termini are conserved domains of the zinc finger motif (N terminus) and nuclear localization signals (C terminus). Arrowheads indicate cysteine residues in the zinc finger domain. Boldface letters represent identical amino acid residues among the three proteins.

(B) Comparison of the C-terminal zinc finger domain of Nipponbare Hd1 and Ginbouzu Se1 (*Hd1*) proteins. Amino acid substitutions and deletions are indicated by underlines.

analysis is difficult, because allelic differences in other genes involving heading date are also present. Given the differences between days to heading of Nipponbare (116 ± 1.9) and NIL(*Hd1*) (97.2 ± 1.3) and between Ginbouzu (125.2 ± 1.5) and HS66 (95.2 ± 2.2), we assume that the inhibition of heading under natural field conditions by the *Hd1* allele of Nipponbare is less than that of Ginbouzu. We also observed a difference in days to heading between two *se1* mutants, HS66 (95.2 ± 2.2) and HS110 (103.8 ± 1.9). In the RT-PCR assay, we detected a normal-size *Hd1* transcript in HS110. These results suggest that the function of the *se1* (*hd1*) allele of HS110 is not completely lost. It will be necessary to confirm the functional difference between Nipponbare and

Ginbouzu *Hd1* alleles and two mutant alleles of *se1*(*hd1*) by transformation analysis.

Function of *Hd1* in Photoperiod Response

The function of *Hd1* probably is to affect transcription activation because of the presence of a zinc finger domain. Because *Hd1* transcription itself was not greatly affected by change in length of day, we speculate that *Hd1* affects the transcription of genes for which expression is controlled by photoperiod changes. A nearly isogenic line for *Hd1*, in which the Kasalath *Hd1* chromosomal region was substi-

tuted into the Nipponbare background, was developed in a previous study (Lin et al., 2000). *NIL(Hd1)* exhibited later heading than Nipponbare under SD conditions but earlier heading under LD conditions (Lin et al., 2000). We analyzed naturally occurring allelic variation—whether such genes promote or inhibit heading under certain photoperiod conditions—in contrast with mutants because the functional parental alleles were uncertain. In the case of *Hd1*, high-resolution linkage mapping clearly suggested that a functional allele of *Hd1* inhibited heading under natural field conditions (more like LD conditions in the growth chamber). On the other hand, transformation analysis revealed that *Hd1* promoted heading under SD conditions. These results suggest that *Hd1* might be bifunctional under SD and LD conditions, promoting heading under SD conditions and inhibiting it under LD conditions. The fact that the same amount of *Hd1* mRNA was present under both SD and LD conditions also supports this hypothesis. In *Arabidopsis*, the *CO* protein promotes flowering under LD conditions but has no phenotypic effect on flowering time under SD conditions (Putterill et al., 1995). This raises the question of what factors are involved in regulating the marked change in photoperiod response from SD to LD conditions. In *Arabidopsis*, one possible explanation is derived from expression analysis of *CO* and *GI* genes (Samach and Coupland, 2000). The daily *GI* expression peak occurred 8 to 12 hr after dawn, and the timing and duration of the peak were influenced by length of day (Fowler et al., 1999). This change of expression pattern might be responsible for the photoperiod response in *Arabidopsis*. In this study, we analyzed mRNA at a single time during daylight (5 hr after dawn). More comprehensive expression analysis of rice *Hd1*, such as measurement of daily temporal expression pattern, should be performed under conditions of different photoperiod lengths.

Genetic Control of PS in Rice

The genetic control pathway of rice heading has not been modeled successfully. Even though the importance of phytochrome in the photoperiod response has been recognized in other plant species, only recently was it proved to play an important role in rice (Izawa et al., 2000). Other factors involved in photoperiod response in rice are still uncertain. To clarify the genetic control of heading in rice in detail, we have been genetically analyzing naturally occurring variation (Yano and Sasaki, 1997; Yano et al., 1997; Lin et al., 1998; Yamamoto et al., 1998, 2000). Nonfunctional alleles of *Hd1* and other genes at QTLs for photoperiod response were combined. Analysis of epistatic interactions revealed that *Hd1* is epistatic to other genes that enhance photoperiod response, such as *Hd2* and *Hd3* (Lin et al., 2000). These results suggest that *Hd1* plays a central role in the expression of photoperiod response under both SD and LD conditions. However, this study suggests that the transcription of *Hd1* might not be greatly affected by changes in photoperiod

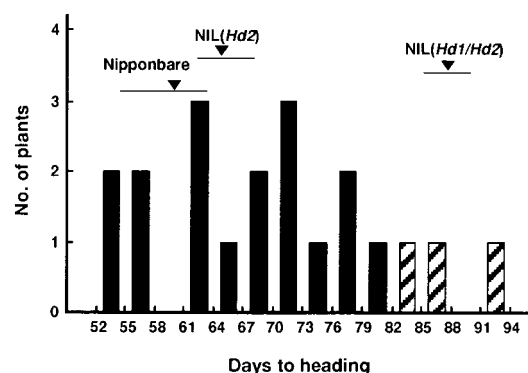


Figure 4. Frequency Distribution for Days to Heading in Self-Pollinated Progeny (T_1) of One 7.1-kb Transformant.

All plants were cultivated under SD conditions (10.0 hr) in a controlled growth chamber. Bars indicate plants with (black) and without (hatched) the candidate genomic fragment. *NIL(Hd1/Hd2)* is the recipient line for transformation, and *NIL(Hd2)*, the nearly isogenic line of *Hd2*, can be a control of transformation when the functional *Hd1* is complemented in *NIL(Hd1/Hd2)*. Means and ranges of days to heading of Nipponbare, *NIL(Hd1/Hd2)*, and *NIL(Hd2)* are indicated by arrowheads and horizontal bars, respectively.

length. These observations raise a major question: which factors are involved in the dramatic change in the response to photoperiod in rice? We cannot rule out the hypothesis that *Hd1* itself is involved in such a strong response. However, other factors for which expression is affected by changes in photoperiod might be involved downstream of *Hd1* in the genetic control pathway. To clarify the real role of *Hd1* in the photoperiod response pathway, we need to analyze rhythmic expression under SD and LD conditions. In addition, identification of other QTLs, such as *Hd2*, *Hd3*, and *Hd6*, by map-based cloning is progressing (Yano and Sasaki, 1997; Yamamoto et al., 2000; Y. Takahashi and M. Yano, unpublished data). This simultaneous approach to identifying QTLs that control PS will contribute to our understanding of the genetic control pathway for photoperiod response in rice.

This study proves that genes with the same structure are involved in flowering in the dicot species *Arabidopsis* and the monocot species rice. A casein kinase II α subunit also is involved in the photoperiod response of flowering in rice (Y. Takahashi and M. Yano, unpublished data). The casein kinase II activates the CCA1 protein, a circadian oscillator in *Arabidopsis* (Sugano et al., 1998), and is involved in the control of flowering (Sugano et al., 1999). These results clearly indicate that the same factors (genes) might be involved in photoperiod response in both SD plants (rice) and LD plants (*Arabidopsis*). However, we do not understand the mechanism that makes each plant species completely opposite in their photoperiod responses. Our efforts to identify

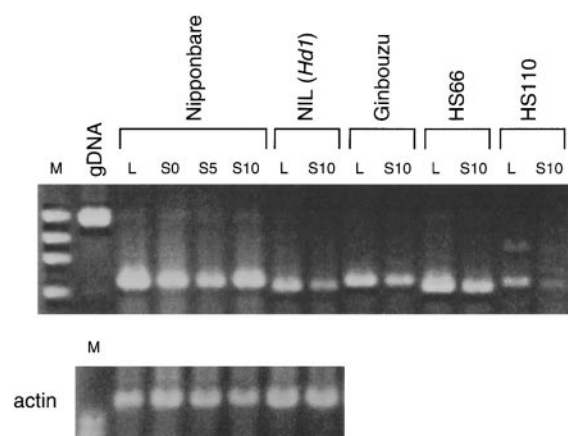


Figure 5. Detection of mRNA in the Varieties and Lines Used in This Study by RT-PCR Assay.

All plants were raised in LD conditions (16.0 hr) and then subjected to the following treatments: L, additional 10 days of treatment in LD conditions; S0, no additional treatment; S5, additional 5 days of treatment in SD conditions (10.0 hr); S10, additional 10 days of treatment in SD conditions. The actin control is shown at the bottom of figure. Lane M indicates DNA size markers. Lane gDNA is a PCR product of genomic DNA of Nipponbare as a template.

genes in rice by using both naturally occurring allelic variation and mutational analysis might provide important clues to the mechanisms of these phenomena.

METHODS

Plant Materials

Rice (*Oryza sativa*) varieties Nipponbare and Kasalath were used to develop a large mapping population. Self-pollinated progeny (BC_3F_3), derived from BC_3F_2 plants heterozygous for the proximal region of chromosome 6 (including the *Hd1* target locus), were used as the segregating population. The BC_3F_2 plants used in small-scale mapping of *Hd1* were described previously (Yamamoto et al., 1998). Six varieties or lines were used in genomic DNA gel blot hybridization analysis, reverse transcription-polymerase chain reaction (RT-PCR) assay, and sequencing of the genomic candidate region. A nearly isogenic line for *Hd1* (NIL[*Hd1*]), in which the Kasalath chromosomal region of *Hd1* was substituted into the genetic background of Nipponbare, was developed previously (Lin et al., 2000). Artificial mutants for the *Se1* locus, HS66 and HS110, were induced by gamma irradiation from the variety Ginbouzu at the Plant Breeding Laboratory at Kyoto University (Inoue et al., 1992). These rice varieties and lines were cultivated in controlled growth chambers or under natural field conditions. Leaves were collected for DNA and RNA extraction at the appropriate stages (see legend to Figure 5). Nipponbare and Ginbouzu showed a strong photoperiod response, but Kasalath

showed a weak response. Days to heading of Nipponbare, NIL(*Hd1*), Ginbouzu, and the *se1* mutants HS66 and HS110 under natural field conditions are stated in Results, except for Kasalath (105.2 ± 1.3 days).

Growth Conditions for Expression Analysis

All varieties, mutants, and the nearly isogenic line were grown in growth chambers with different photoperiod conditions at temperatures of 26°C for 12 hr and 22°C for 12 hr. Two photoperiod treatments were used: short day (SD; 10.0 hr) and long day (LD; 16.0 hr). Photon irradiance was $\sim 700 \mu\text{mol m}^{-2} \text{sec}^{-1}$. The plants were grown under LD conditions for 30 days, after which some were transferred to SD conditions. Plants were sampled at 30 and 40 days under LD conditions and at 5 and 10 days under SD conditions. Sampling time was ~ 5 hr after dawn.

High-Resolution Mapping

Approximately 9000 BC_3F_3 plants were grown in a field at the National Institute of Agrobiological Resources. Segregants homozygous for the Kasalath *Hd1* allele were selected based on days to heading (early heading). The selected plants were used for recombination detection in the genomic region flanking *Hd1* by the pooled sampling method (Churchill et al., 1993). Green leaves of five individuals were pooled for DNA extraction by the cetyltrimethylammonium bromide method (Murray and Thompson, 1980). DNA was digested with the restriction enzymes BamHI, BglII, HindIII, and EcoRI for restriction fragment length polymorphism (RFLP) analysis. DNA gel blotting, probe production, labeling, and detection were performed as described previously (Kurata et al., 1994; Harushima et al., 1998). Two probes, R1679 and P130, which flanked *Hd1*, were used to detect recombination events between *Hd1* and the markers. When a particular pool of five plants was found to contain a recombinant chromosome, the five plants were analyzed individually to identify the plant with the recombinant chromosome. The total DNA of each BC_3F_3 plant was reconstituted from the bulked DNA of BC_3F_4 seedlings. Approximately 100 BC_3F_4 seedlings were grown in a greenhouse, and the leaves were harvested for DNA extraction. RFLP analysis was performed as described above. Plants with a crossover between the flanking markers and *Hd1* were used for further fine mapping with additional RFLP and cleaved amplified polymorphic sequence (CAPS) markers.

Screening of Genomic Clones Containing the *Hd1* Locus

A rice P1-derived artificial chromosome (PAC) library (inserts averaging 112 kb from $\sim 70,000$ clones) was constructed with genomic DNA of Nipponbare at the Rice Genome Research Program (RGP; Baba et al., 2000). From this library, 18,432 clones were screened by PCR with sequence tag site primers designed for markers S2539, S20481, and Y4836R. To obtain sequences of the corresponding Kasalath candidate region for *Hd1*, we screened a cosmid library constructed with Kasalath genomic DNA. The genomic library was constructed using the superCos vector (Stratagene, La Jolla, CA). Flanking markers S2539 and S20481 were used to screen the library.

Generation of New DNA Markers Flanking *Hd1*

To produce additional DNA markers flanking the *Hd1* locus, we cloned the end sequence of Y4836R. Yeast artificial chromosome end DNA fragments were amplified by a slightly modified cassette PCR method (Isegawa et al., 1992) and were cloned with the TA vector (Invitrogen, Carlsbad, CA). CAPS markers were generated to narrow the minimum candidate region for *Hd1*. From the sequence data of the candidate region in Nipponbare, we designed several primer pairs to amplify specific amplified products of the target region. The amplified products were digested with 28 restriction enzymes to search for polymorphisms. When we could not find an appropriate enzyme for polymorphisms, we cloned and sequenced the corresponding genomic region in Kasalath. Using sequence comparison, we then found an appropriate restriction enzyme to produce polymorphisms between Nipponbare and Kasalath.

Sequence Analysis

The shotgun sequencing strategy was used to obtain genomic sequencing data covering the *Hd1* locus. The insert of PAC clone P0038C5 was purified by ultracentrifugation and fragmented by ultrasonication, and the ends of each piece were blunt-ended with T4 DNA polymerase. After electrophoresis on agarose gels, fractions corresponding to 2 and 5 kb were cut out, and the eluted DNA fragments were ligated to pUC18 to make sublibraries. Sequencing was performed with an automated fluorescent laser sequencer and a Big-Dye Primer Cycle Sequencing Kit (Perkin-Elmer Applied Biosystems, Foster City, CA). The restriction enzymes *Pst*I and *Avr*II were used to subclone Kasalath cosmid clone no. 47. If necessary, additional restriction enzymes were used to obtain subclones of the appropriate insert size. Sequencing was performed as described above. The raw sequence data of the PAC insert were scored with phred software (Ewing et al., 1998) and then assembled with phrap/cross-match software to make a contiguous nucleotide sequence (Ewing and Green, 1998).

The sequence of the candidate genomic region was analyzed with Genscan software (Burge and Karlin, 1997). A maize model was used to predict putative open reading frames in the candidate region. The predicted amino acid sequence was used for BLAST searches (Altschul et al., 1990) of nonredundant protein databases at GenBank.

Complementation Test for the Function of the Candidate Genomic Region

A 7.1-kb *Apal* fragment containing the candidate *Hd1* region was subcloned into binary vector pZP2H-lac, a plasmid vector without promoter (T. Fuse and M. Yano, unpublished data). An *Agrobacterium*-mediated method was used for transformation (Toki, 1997). Subclones of the 7.1-kb fragment were transformed into *Agrobacterium* strain EHA101 and then infected into callus of a nearly isogenic line of Nipponbare (NIL[*Hd1/Hd2*]), in which the chromosomal regions of the photoperiod sensitivity (PS) loci *Hd1* and *Hd2* had been replaced with chromosomal segments of Kasalath; the resulting nearly isogenic line showed no photoperiod response (Lin et al., 2000). Plants regenerated from hygromycin-resistant calluses were grown in the controlled growth chamber under SD conditions (10.0 hr). Self-pollinated progeny of transformants were grown there under

the same conditions. Days to heading of individual plants were scored and used for confirmation of cosegregation of both heading date and the integrated gene.

RNA Extraction and RT-PCR Assay

Total RNA was extracted from leaves at several stages under various photoperiod treatments in a single-step method (Chomczynski and Sacchi, 1987) with minor modifications. A first-strand cDNA was synthesized with the First-Strand cDNA Synthesis Kit (Amersham Pharmacia Biotech, Uppsala, Sweden) according to the manufacturer's instructions. The first-strand cDNA was used as a template, and amplification was performed for 30 PCR cycles (1 min at 94°C, 2 min at 55°C, 3 min at 72°C) followed by 7 min at 72°C. The primers for *Hd1* were 5'-TTCTCCTCTCCAAGATTCC-3' (sense) and 5'-CATACG-CCTTTCTTGTTTCA-3' (antisense). Actin primers were 5'-TCC-ATCTTGGCATCTCTCAG-3' (sense) and 5'-GTACCCGCATCAGGC-ATCTG-3' (antisense). RT-PCR assay was performed at least twice for each sample. Rapid amplification of cDNA ends PCR was performed by using a Marathon cDNA Amplification Kit (Clontech, Palo Alto, CA). Amplification using reduced cycles in the RT-PCR assay was performed to confirm the results obtained.

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