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ORIGINAL PAPER

Complete loss of photoperiodic response in the rice mutant line X61 is caused by deficiency of phytochrome chromophore biosynthesis gene

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Abstract In rice (*Oryza sativa*), a short-day plant, photoperiod is the most favorable external signal for floral induction because of the constant seasonal change throughout the years. Compared with Arabidopsis, however, a large part of the regulation mechanism of the photoperiodic response in rice still remains unclear due mainly to the lack of induced mutant genes. An induced mutant line X61 flowers 35 days earlier than its original variety Gimbozu under a natural photoperiod in Kyoto (35°01′N). We attempted to identify the mutant gene conferring early heading to X61. Experimental results showed that the early heading of X61 was conferred by a complete loss of photoperiodic response due to a novel single recessive mutant gene *se13*. This locus interacts with two crucial photoperiod sensitivity loci, *Se1* and *E1*. Wild type alleles at these two loci do not function in coexistence with *se13* in a homozygous state, suggesting that *Se13* is an upstream locus of the *Se1* and *E1* loci. Linkage analysis showed that *Se13* is located in a 110 kb region between the two markers, INDEL3735_1 and INDEL3735_3 on chromosome 1. A database search suggested that the *Se13* gene is identical to AK101395 (=*OsHY2*), which encodes phytochromobilin synthase, a key enzyme in phytochrome chromophore biosynthesis. Subsequent sequence analysis revealed that X61 harbors a 1 bp insertion in exon 1 of *OsHY2*, which induces a frameshift mutation producing a premature stop codon. It is therefore considered that the complete loss of photoperiodic response of X61 is caused by a loss of function of the *Se13* (*OsHY2*) gene involved in phytochrome chromophore biosynthesis.

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Introduction

Flowering time is an important characteristic closely associated with the regional adaptability of plants. Plants start floral bud formation in response to environmental signals, such as photoperiod and temperature. Among these signals, photoperiod is the most favorable for floral induction because its seasonal change is constant among years, whereas the other signals vary among years and fields. Therefore, a large majority of plant species, except those originated in low latitudes, have evolved mechanisms to integrate photoperiod information into their developmental programs for floral induction.

Recent molecular genetic analyses of the long-day plant Arabidopsis (*Arabidopsis thaliana*) have accumulated information about the genetic, physiological, and biochemical mechanisms of the photoperiodic response for floral induction (Yanovsky and Kay 2002; Valverde et al. 2004; Kobayashi and Weigel 2007; Turck et al. 2008). In most of those studies, flowering-time and flower-formation mutants induced from various ecotypes have been utilized, which indicates the usefulness and significance of induced mutations in elucidating the physiological and biochemical mechanisms of flowering (Koornneef et al. 1998; Reeves and Coupland 2001).

In the short-day plant rice (*Oryza sativa* L.), conventional genetic analyses of flowering time (=heading time)

have been extensively performed; consequently, many loci involved in the photoperiod response (photoperiod sensitivity) have been identified (Yokoo et al. 1980; Yamagata et al. 1986; Poonyarit et al. 1989; Sano 1992; Yokoo and Okuno 1993; Tsai 1995). Among them, two major photoperiod sensitivity loci, *Se1* (*Photoperiod sensitivity 1*) and *E1* (*Heading time 1*), have played an important role in the regional differentiation of rice varieties (Yokoo et al. 1980; Ichitani et al. 2002; Okumoto et al. 1996). Recent molecular genetic analyses of rice heading time genes revealed that the genetic photoperiod-sensitive pathway to heading in rice is partly common to that of Arabidopsis (Izawa et al. 2003; Hayama and Coupland 2004). The Arabidopsis gene *CO* (*CONSTANS*) promotes flowering under long photoperiods (LPs), whereas the rice gene *Se1* (=*Hd1*), a *CO* ortholog in rice, promotes and represses heading under short photoperiods (SPs) and LPs, respectively (Yano et al. 2000). More recently, *Grain height and heading date 7* (*Ghd7*) encoding a CCT (CO, CO-LIKE, and TIMING OF CAB1)-domain protein was isolated from natural variations in rice (Xue et al. 2008). *Ghd7* also represses heading under LPs and is known to be identical to the *E1* locus (Saito et al. unpublished data). Despite the recent, rapid accumulation of knowledge about physiological and biochemical functions for floral induction in rice, a large part of the regulation mechanism remains unknown. This is because few mutant genes suitable for such investigations have yet been identified. To exploit new loci for heading time, Tanisaka et al. (1992) induced many mutants. Of these mutants, X61 exhibits heading as many as 35 days earlier than the original variety Gimbozu under a natural photoperiod in Kyoto (35°01′N), Japan.

In the present study, we first analyzed the inheritance of the extremely early heading of X61 and its photoperiodic response to three different photoperiods. Since X61 proved to be a single recessive mutant line of the original variety, we investigated the interactive effects of the mutant locus with two crucial photoperiod sensitivity loci, *Se1* (=*Hd1*) and *E1* (=*Ghd7*), and the chromosomal location of the mutant gene. Consequently, we found that the mutant gene is a novel one involved in phytochrome chromophore biosynthesis. The mutant gene is valuable and helpful for understanding photoperiodic response mechanisms in rice and also for the future development of photoperiod insensitive rice varieties.

Materials and methods

Analysis of photoperiodic response

The extremely early heading mutant line X61 and its original variety Gimbozu were used. X61 was induced by gamma-irradiation of Gimbozu seeds. The genotypes for the heading-time genes are shown in Table 1. Seeds were disinfected by soaking in a benlate—methyl-1-[(buthylamino) carbonyl]-*H*-benzimidazol-2-ylcarbamate—solution (diluted with water 1,000-fold) at 20°C for 24 h, and were pre-germinated by soaking in water at 30°C for 2 days. Ten seeds were sown on field soil in a 3.6-L pot, and were covered with granulated soil. Seedlings were thinned to 5 plants per pot 14 days after sowing. All the tillers, except for the main culm, were cut off whenever they reappeared. Every other week, 20 mL 0.2% (w/v) Hyponex (mixed fertilizer including 6% nitrogen, 10% water-soluble phosphoric acid and 5% water-soluble potassium; HYPONex Co. Ltd, Osaka, Japan) solution was applied to each pot as additional fertilizer.

Table 1 Days to heading and genotypes for heading time genes of varieties/lines used in this study

Variety/line	Days to heading[a]	Genotype		
		Se1 locus	*E1* locus	*Se13* locus[b]
Gimbozu	80.4	*Se1*	*E1*	*Se13*
X61	49.4	*Se1*	*E1*	*se13*
Kasalath	76.2	*se1*	*E1*	*Se13*
SL6	80.7	*Se1*	*E1*	*Se13*
HS110	64.5	*se1*	*E1*	*Se13*
EG2	69.5	*Se1*	*e1*	*Se13*
DM61_2	42.9	*Se1*	*e1*	*se13*
DM61_110	51.3	*se1*	*E1*	*se13*

All the variety/lines except Kasalath have the same genotypes for other flowering-time loci

[a] The varieties and lines were grown under long photoperiod (14 h) condition

[b] Mutant locus identified in this study (*Se13*, wild-type allele; *se13*, mutant allele)

Three photoperiods of 10 (SP), 14 (LP) and 24 h, beginning at the sowing date, were used. Photoperiod treatments were conducted using three growth cabinets without temperature control. Ten pots of each line were placed under each photoperiod. In addition to natural daylight (8:00–18:00), supplementary artificial light from incandescent lamps (3.24 Wm^{-2} at soil surface) was used for the 14 and 24 h photoperiod treatments. The experiment was conducted from 1 May to early October, 2008. Heading data was recorded for each plant when the first panicle emerged from the sheath of the flag leaf.

Genetic analysis and mapping of the mutant gene(s)

The F_2 population, comprising 315 plants, from the cross between X61 and its original variety Gimbozu were subjected to a genetic analysis for heading time under a natural

photoperiod in Kyoto (35°01′N) in 2005. In Kyoto, the natural photoperiod is longer than the critical photoperiod (approximately 13.5 h) during the cropping from May to the end of July (Nishida et al. 2002), regarded as a long photoperiod condition, and after that it becomes shorter than 13.5 h regarded as a short photoperiod condition (Fig. 1). The progeny test was conducted with 50 F_3 lines. Each F_3 line (about 25 plants/line) was the progeny of the F_2 plant randomly selected from the F_2 population in 2006. In both years, germinated seeds were sown in nursery beds in a green house on 1 May, and seedlings were transplanted to a paddy field in Kyoto 30 days after sowing.

We attempted to identify the chromosomal location of the mutant gene through three steps; a rough mapping with a single-marker QTL analysis, narrowing down the candidate region through a linkage analysis with a chromosome segment substitution line (CSSL), and a fine mapping with additional INDEL (insertion or deletion) markers in the candidate region. Thus we first attempted to make a rough mapping through a single-marker QTL analysis with 46 SSR (simple sequence repeat) markers using 96 F_2 plants of the cross between X61 and the *indica* variety Kasalasth. The number of SSR makers would not be sufficient to detect the QTLs with minor effects. The mutant gene, however, had a great decreasing effect on DH (ca. 35 days). So we thought that even a QTL analysis with a small number of markers could detect the QTL of the mutant gene. The F_2 plants were grown under a natural photoperiod in Kyoto in 2006. X61 harbored a functional allele *Se1* at the *Se1* locus crucially involved in the photoperiod sensitivity of rice, whereas Kasalath harbored a nonfunctional allele *se1* (Yano et al. 2000). The genotypic effect of this locus on DH is quite large in Kyoto: *Se1Se1* flowers about 10 days later than *se1se1*. To exclude such an effect of the *Se1* locus and perform a single-marker QTL analysis efficiently, we classified the F_2 population into three genotypic groups for the *Se1* locus, X61-homozygous, heterozygous and Kasalath-homozygous groups. We designed a PCR primer set (Se1_kg_U1: 5′-GGACTCTTGGCTTCTCCTCTC-3′, Se1_kg_L1: 5′-TGTGAAGGTACCACACACTC-3′) to detect the Kasalath-type allele *se1* harboring a 33 bp deletion in exon 1 (Yano et al. 2000). For the single-marker QTL analysis, QTL cartographer ver. 2.5 (Wang et al. 2005) was used. The 46 SSR markers were chosen so that they were widely deployed in the rice genome.

Subsequently, to narrow down the candidate region of the mutant gene, the F_2 population, comprising 582 plants, from the cross between X61 and SL6 was grown under a natural photoperiod in a paddy field in Kyoto in 2007. SL6 is a chromosome segment substitution line (CCSL) harboring a Kasalath-derived chromosome segment including the candidate region of the mutant gene of X61 on the long arm of chromosome 1 in the Nipponbare background (Yano and Ebitani 2002; see also the Rice Genome Resource Center: http://www.rgrc.dna.affrc.go.jp). To further narrow down the candidate region of the mutant gene, 1,404 recombinants between INDEL4073_2 and RM12212 were grown under a natural photoperiod in a paddy field in Kyoto in 2008 and surveyed with four additional INDEL markers which were designed based on INDEL polymorphism between the *japonica* variety Nipponbare and the *indica* variety 93-11 (Table 2).

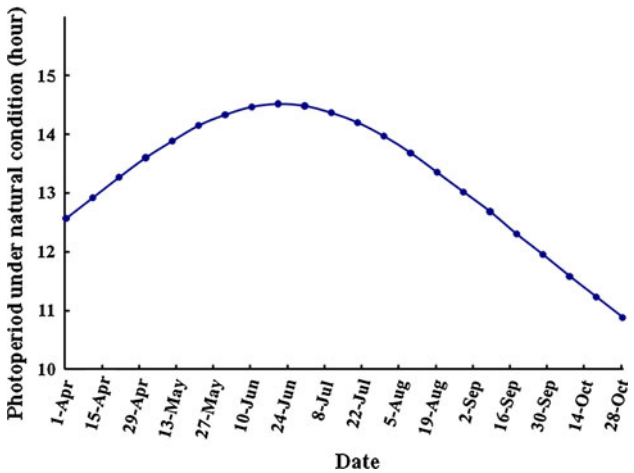

Fig. 1 Seasonal change of natural photoperiod (from sunrise to sunset) in Kyoto, Japan (35°01′N)

Analysis of interactions of the mutant locus with the *Se1* and *E1* loci

Six F_2 populations of the X61/HS110, X61/EG2, X61/DM61_2, EG2/DM61_2, X61/DM61_110 and HS110/

Table 2 Primer sequences of additional PCR markers used for the mapping of the *se13* locus

Marker name	Forward sequence	Reverse sequence
INDEL4073_2	TGTGAGAAGCTGGTGGAA	TGATGTGTAGCCTTGCTTGG
INDEL3683_2	TGCCTTCCTGGATCTGATTT	CTTTGCTATGGCCCACATCT
INDEL3735_1	ACCTTTTGGCTTTGCCTTTT	AACTGGCATCCTTTTGTTGC
INDEL3735_3	AATGCCGTCTCCATCGAC	CTGCAGAGGAGGGAGGTTG

DM61_110 crosses were planted in a paddy field in Kyoto in 2007. HS110, like X61, is an early heading mutant line induced from Gimbozu (ca. 10 days earlier than Gimbozu), harboring a single recessive mutant gene *se1* at the *Se1* locus (Yano et al. 2000). EG2 is a heading-time tester line harboring *e1* at the *E1* locus, and the genotypic difference between EG2 and Gimbozu is only at the *E1* locus (Gimbozu harbors *E1*) (Yamagata et al. 1986; Nishida et al. 2001). DM61_2 and DM61_110 are heading-time tester lines that were developed for this study by introducing *e1* and *se1* genes to the X61 background, respectively. The genotypes for the heading-time genes of these lines are shown in Table 1.

Sequence analysis of the mutant gene

Sequence analysis of the AK101395 gene (=*OsHY2*) in X61 and Gimbozu with the DNA Data Bank of Japan (http://www.ddbj.nig.ac.jp) was performed by using six primer pairs designed from the *OsHY2* genomic sequence of the variety Nipponbare. DNA was extracted from the fresh leaves (about 100–200 mg) of each plant according to Komari's method (1989) with slight modifications. After PCR amplification, the products were separated by electrophoresis on a 2% agarose gel and then the DNA fragments were purified from the agarose gel using a Qiaquick Gel Extraction kit (QIAGEN Sciences, USA). The purified DNA fragments were sequenced using a Dye Terminator Cycle Sequencing (DTCS) Quick Start Kit and a CEQ8000 Genetic Analysis System (Beckman Coulter Inc, Fullerton, CA, USA).

Co-segregation analysis of the mutant gene and the SNP in *OsHY2* gene

We performed dCAPS (derived cleaved amplified polymorphic sequence) analysis to identify the genotype of the *OsHY2* locus in the F_2 and F_3 plants of the X61/SL6 cross. As described later, the X61 allele harbors a digestion site of *Bsi*YI due to a 1 bp insertion, whereas the SL6 allele does not harbor it. A pair of PCR primers (SNP_OsHY2_U1: 5′-GACCACCCTCACCCCTCAACCC-3′, SNP_OsHY2_L1: 5′-TCATCAAACCAAGGGCACCTA-3′) were designed to amplify the 175 bp segment including the SNP site. When digesting the amplicon with *Bsi*YI, the fragment of the X61 allele was shorter than that of the SL6 allele by 25 bp. The amplified products were digested with *Bsi*YI at 37°C for 1 h. The digested amplicons were separated on a 12% acrylamide gel. After electrophoresis, the gel was stained with ethidium bromide and the DNA fragments were visualized under UV light.

Results

Analysis of photoperiodic response

Under a 10 h photoperiod (SP condition), X61 (51.0 DH) showed almost the same DH as that of the original variety Gimbozu (51.3 DH). Under a 14 h photoperiod (LP condition), however, X61 exhibited 49.0 DH, while Gimbozu exhibited 78.2 DH (Fig. 2). Under a 24 h photoperiod, X61 exhibited 49.3 DH, while Gimbozu did not reach the reproductive phase even after 140 days from sowing (Fig. 2). Thus, while Gimbozu showed great differences in DH between photoperiods, X61 exhibited no significant difference between photoperiods. This indicates that X61 looses photoperiod sensitivity completely.

Genetic analysis and mapping of the mutant gene(s)

The F_2 population from the cross between X61 and Gimbozu showed a bimodal distribution within the parental ranges with a clear breakpoint, which divided the population into early (X61 type) and late (Gimbozu type) groups (Fig. 3). The early type to late type ratio fit the 1:3 ratio expected for one-locus segregation ($\chi^2 = 1.978$, $P > 0.05$). In the progeny test, all the 50 F_3 lines could easily be classified into three groups. The 11:29:10 ratio for [X61 type] to

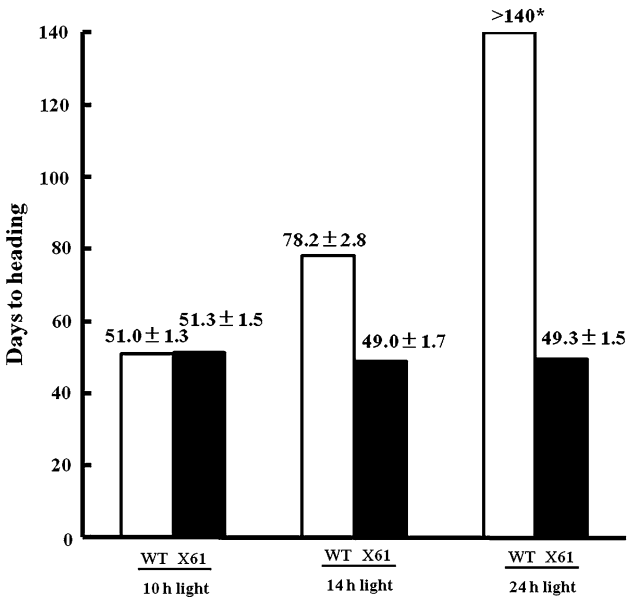

Fig. 2 Days to heading (DH) of Gimbozu wild type (WT) and X61 under different photoperiod conditions. Heading date was recorded under 10 (short photoperiod), 14 (long photoperiod), and 24 h photoperiods (constitutive daylight condition). The number above the *chart* indicates the average DH with the standard error. *Asterisk* indicates that plants did not reach heading. Experiments ended 140 days after sowing because the average temperature was dropping below 20°C

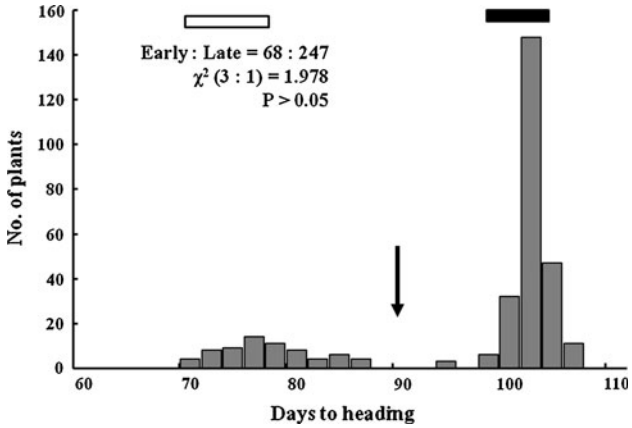

Fig. 3 Frequency distribution for days to heading (DH) in the F_2 population from the Gimbozu/X61 cross. *White* and *black bars* indicate the ranges of DH of X61 and Gimbozu, respectively. *Arrow* indicates the breakpoint between early heading plants and late heading plants

[segregating type] to [Gimbozu type] lines fit the 1:2:1 ratio ($\chi^2 = 0.517$, $P > 0.05$) expected for one-locus segregation. This indicates that the early heading of X61 is conferred by a single recessive mutant gene. We designated this gene *se13* (a photoperiod insensitive allele).

The F_2 population, comprising 96 plants, from the X61/Kasalath cross showed a continuous distribution with many transgressive segregants (data not shown). We divided the F_2 population into three genotypic groups for the *Se1* locus, and performed a single-marker QTL analysis with 46 SSR markers for every genotypic group. Consequently, a total of seven QTLs were detected (Table 3). Among them, four with a positive effect (longer DH) in the X61-type allele were excluded from the candidate of the mutant gene, because X61 was an extremely early heading time mutant. To the contrary, the remaining three, RM5536 on the long arm of chromosome 1, RM38 on the short arm of chromosome 8, and RM3181 on the short arm of chromosome 8, showed a significant negative effect (shorter DH) in the X61-type allele (Table 3). Among the three, RM38 and RM3181 were closely linked to the known heading time QTL *Hd5*, which was identified in the F_2 population of the Nipponbare/Kasalath cross (Yano et al. 1997; Lin et al. 2003). Monden et al. (2009) reported that one major QTL on the short arm of chromosome 6 was contributory to the DH difference between Gimbozu and Nipponbare, indicating that those two varieties have the same genotypes for heading time except this major QTL. Accordingly, RM38 and RM3181 should be detected also in the Gimbozu/Kasalath cross. X61 is a single recessive mutant line from Gimbozu. Therefore, the flanking regions of RM38 and RM3181 on chromosome 8 could be excluded from the candidate region of the mutant gene. Thus we considered that the mutant gene was closely linked to RM5536 on chromosome 1.

Subsequently, we attempted to make a fine mapping of the *Se13* locus with additional four SSR markers and two INDEL markers, using 582 F_2 plants of the X61/SL6 cross. Experimental results demonstrated that the chromosomal location of the *Se13* locus was located in the region with a physical distance of <474 kb between the two markers, INDEL4073_2 and RM12212, on chromosome 1 (Fig. 4a). Further analysis was conducted by using 1404 F_3 plants from the recombinants (F_2 plants) between INDEL4073_2 and RM12212. Consequently, the candidate region of the *Se13* locus was narrowed down in the region between INDEL3735_1 and INDEL3735_3 with a physical distance of <110 kb (Fig. 4b; Table 4). Since no heading time gene controlling photoperiod sensitivity has been reported in this region, we consider this mutant gene *se13* to be novel.

Table 3 The results of a single-marker QTL analysis for each genotypic group

Mapping population[a]	Chr.	Marker	LR[b]	*P* value	Additive effect	Allelic mean (days) ± SD[c]		Referenced QTL[d]
						X61	Kasalath	
X61 homozygote	1	RM5536	6.01	0.022	−12.38	95.0 ± 21.6	118.3 ± 6.7	–
	8	RM3181	5.16	0.034	−8.65	102.2 ± 19.2	119.0 ± 6.7	*Hd5*
	12	RM8215	4.73	0.043	9.42	120.0 ± 2.1	101.5 ± 5.0	*Hd13*
Kasalath homozygote	1	RM243	4.78	0.038	5.54	90.2 ± 6.1	79.5 ± 7.8	–
	8	RM38	5.41	0.028	−6.09	82.8 ± 10.5	95.7 ± 6.8	*Hd5*
Heterozygote	1	RM24	7.77	0.007	8.53	113.3 ± 7.0	97.9 ± 11.6	–
	1	RM5536	5.79	0.019	−7.72	86.9 ± 19.8	104.7 ± 12.2	–
	3	RM6883	12.93	<0.001	10.49	109.9 ± 10.6	90.1 ± 13.9	*Hd9*

[a] Mapping population indicates genotype group of the *Se1* locus

[b] LR indicates likelihood ratio test statistic

[c] SD indicates standard division

[d] The referenced QTLs are reported in Yano et al. (1997)

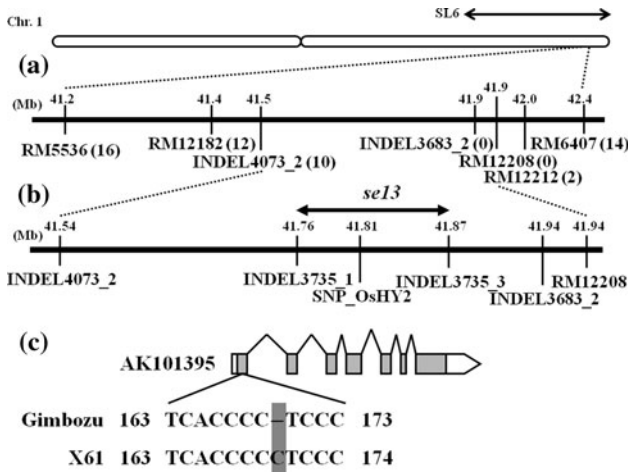

Fig. 4 Chromosomal location of the mutant gene *se13* of X61 and the molecular marker positions on chromosome 1. *Arrow* above the figure indicates the genomic region substituted by a Kasalath chromosome segment in SL6. **a** The result of a fine mapping of *se13* using the F_2 population of the X61/SL6 cross. Number in the *parenthesis* indicates the number of recombinant plants. **b** The result of a fine mapping of *se13* using the F_3 lines. **c** Schematic representation of the genomic sequence of the *OsHY2* gene. *Open* and *gray boxes* indicate the UTR region and CDS region, respectively. The numbers beside the sequences indicate the nucleotide positions from the transcription start site

Analysis of interactions of the mutant locus with the *Se1* and *E1* loci

Using the F_2 populations from the X61/EG2 and X61/HS110 crosses, we investigated the presence of interactions of the *Se13* locus with the *E1* and *Se1* loci. Among the 247 F_2 plants of the X61/EG2 cross, 31 were the X61 type, 54 were the EG2 type, 162 were late transgressive segregant type, and none was the early transgressive segregant type (Fig. 5a). If there was no interaction between *Se13* and the *E1* loci, early transgressive segregants with the genotype *se13se13/e1e1* should have appeared. It is therefore clear that the *Se13* locus interacts with the *E1* locus. To confirm this, X61 (*E1E1/se13se13*) and EG2 (*e1e1/Se13Se13*) were crossed with DM61_2 (*e1e1/se13se13*). The F_2 population from the EG2/DM61_2 showed a bimodal distribution without any transgressive segregants, indicating *Se13* locus segregation (Fig. 6a). The segregation ratio of early to late (41:180) fit the 1:3 ratio ($\chi^2 = 4.900$, $0.01 < P < 0.05$). In contrast, the F_2 populations of the X61/DM61_2 cross showed a continuous but narrow distribution without any transgressive segregants (Fig. 6b), suggesting that the recessive homozygote for the *Se13* locus masked the effect of the functional allele *E1* at the *E1* locus. On the other hand, among 310 F_2 plants of the X61/HS110 cross, 60 were of the X61 type, 60 were the HS110 type, 190 were the late transgressive segregant type, and none was the early transgressive segregant type (Fig. 5b). This suggests the presence of an interaction between *Se13* and the *Se1* locus. To confirm this, X61 and HS110 were crossed with DM61_110. The F_2 population of the HS110/DM61_110 cross showed a bimodal distribution without any transgressive segregants (Fig. 6c). The segregation ratio of early to late (15:56) fit the 1:3 ratio ($\chi^2 = 0.568$. $0.25 < P < 0.50$). This indicates that only the *Se13* locus

Table 4 Marker genotypes and days to heading of the representative F_3 plants

Representative F_3 recombinant plant	Marker genotype[a]						Days to heading	Heading type
	INDEL4073_2	INDEL3735_1	OsHY2_dCAPS	INDEL3735_3	INDEL3683_2	RM12208		
XF3_8_5	SL	J	J	J	J	J	59	Early
XF3_6_7	SL	SL	J	J	J	J	59	Early
XF3_10_1	SL	SL	SL	J	J	J	77	Late
XF3_9_6	SL	SL	SL	SL	J	J	83	Late
XF3_8_20	SL	SL	SL	SL	SL	J	81	Late
XF3_17_10	J	SL	SL	SL	SL	SL	82	Late
XF3_4_174	J	J	SL	SL	SL	SL	77	Late
XF3_4_3	J	J	J	SL	SL	SL	64	Early
XF3_16_42	J	J	J	J	SL	SL	61	Early
XF3_16_29	J	J	J	J	J	SL	60	Early
X61	J	J	J	J	J	J	59	Early
SL6	SL	SL	SL	SL	SL	SL	86	Late

[a] J and SL indicate X61 homozygous and SL6 homozygous, respectively

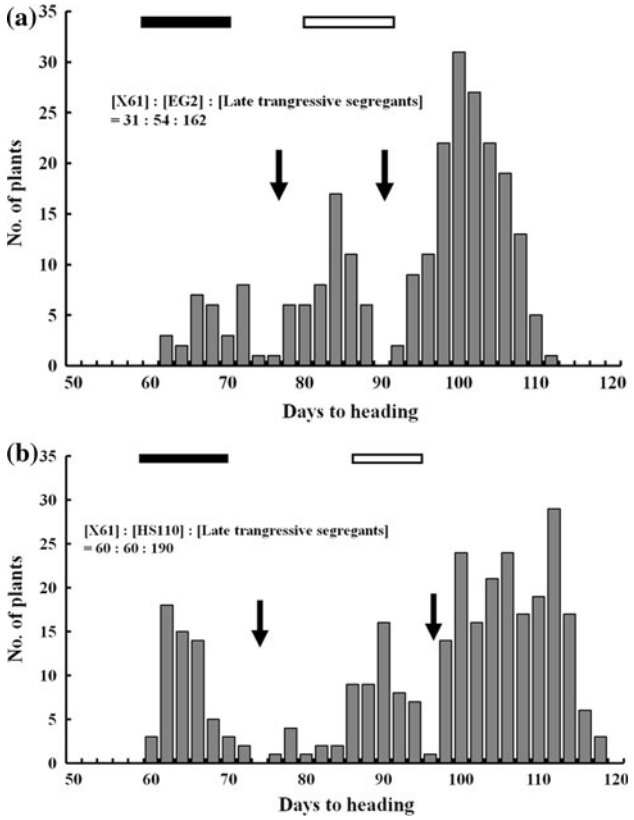

Fig. 5 Frequency distributions for days to heading (DH) in the F_2 populations from the X61/EG2 (**a**) and X61/HS110 (**b**) crosses. *White* and *black bars* indicate the ranges of DH in X61, EG2 (**a**) and HS110 (**b**). *Arrows* indicate the breakpoints between X61 type, EG2 type, and late transgressive segregants (**a**), and between X61 type, HS110 type and late transgressive segregants (**b**)

segregated in this cross combination. In contrast, the F_2 populations from the X61/DM61_110 cross showed a continuous but narrow distribution without any transgressive segregants, suggesting that the recessive homozygote for the *Se13* locus masked the effect of a functional allele at the *Se1* locus (Fig. 6d). Taken together, these results indicate that *Se13* functions as an upstream regulator of the *Se1* and *E1* loci.

Sequence analysis for *OsHY2*

According to the Rice Annotation Project Database (RAP-DB) (http://rapdb.lab.nig.ac.jp/: IRGSP build 4), the 110 kb genomic region contains 11 genes supported by full-length cDNA. Among them, the AK101395 gene (=*OsHY2*) was likely to be the only one involved in photoperiod sensitivity and to be a candidate for the *Se13* gene. Arabidopsis *HY2* encodes phytochromobilin (PΦB) synthase that functions in chromophore biosynthesis. Sequence analysis revealed that X61 harbored a 1 bp insertion in exon 1 of *OsHY2*, which caused a frame-shift mutation producing a premature stop codon in exon 2 (Fig. 4c). This indicates that the *Se13* locus is *OsHY2* most likely, and the deficiency of the phytochrome chromophore due to the frame shift mutation of *OsHY2* causes complete loss of photoperiodic response.

Co-segregation analysis between *Se13* and the SNP in *OsHY2* gene

We attempted to ascertain if the *Se13* locus co-segregates with the SNP in *OsHY2* by dCAPS analysis using the F_2

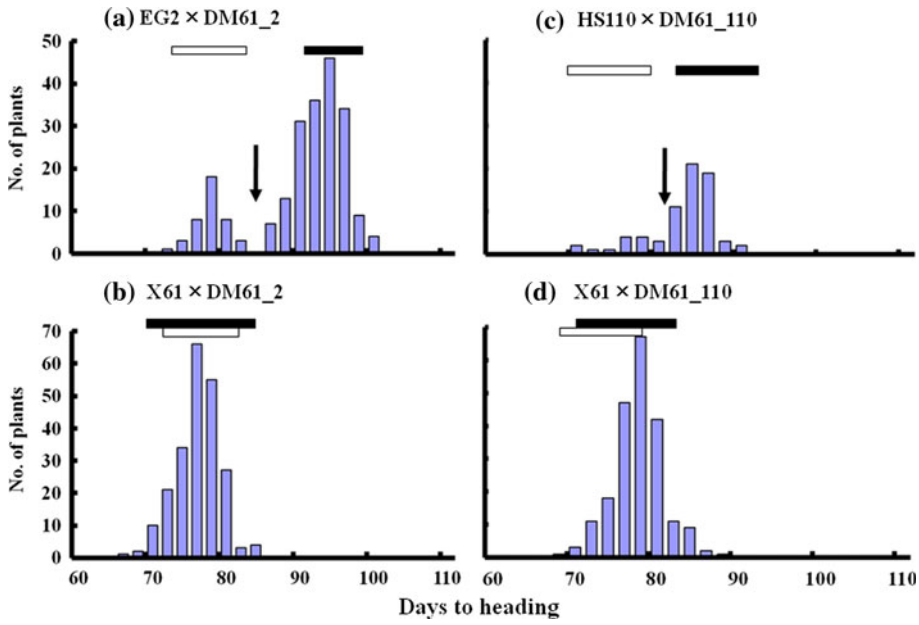

Fig. 6 Frequency distributions for days to heading (DH) in the F_2 populations of the X61/DM61_2 (**a**), EG2/DM61_2 (**b**), X61/DM61_110 (**c**) and HS110/DM110_61 crosses. *Black* and *white bars* indicate the ranges of DH in female and male parents, respectively. *Arrows* indicate the breakpoint between early type and late type plants

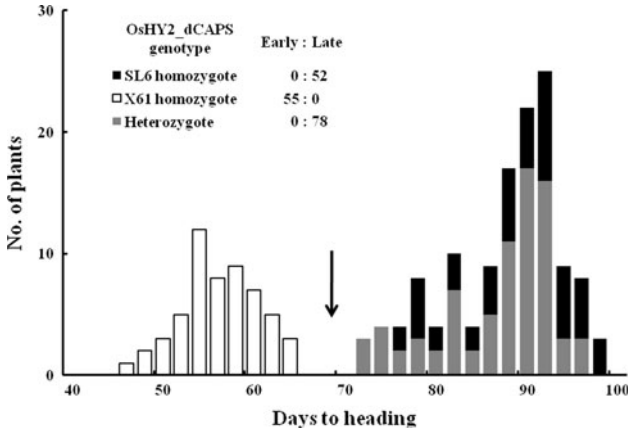

Fig. 7 Frequency distributions for days to heading in the representative F_2 plants of the X61/SL6 cross. *Arrow* indicates the breakpoint between early heading plants and late heading plants

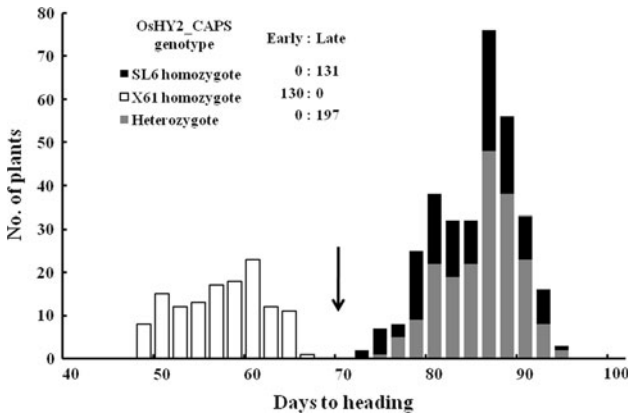

Fig. 8 Frequency distributions for days to heading in the representative F_3 lines of the X61/SL6 cross. *Arrow* indicates the breakpoint between early heading plants and late heading plants

(185 plants) and F_3 populations (718 plants) of the X61/SL6 cross. The F_2 population showed a bimodal distribution within the parental ranges with a clear break point dividing the population into early (X61 type) and late (SL6 type) groups (Fig. 7). The dCAPS analysis showed that all early heading plants harbored the X61 type allele in a homozygous state, whereas all late heading plants either harbored the SL6 type allele in a homozygous state or showed heterozygous type. The F_3 plants from the early heading F_2 plants with the X61-type allele in a homozygous state all exhibited early heading, whereas those from late heading with a SL6-type allele all showed late heading (data not shown). In addition, no recombinant between heading time and *OsHY2* gene was found in the F_3 population. The F_3 population, comprising 458 F_3 plants, derived from the F_2 plants heterozygous for *OsHY2* locus showed a bimodal distribution with a clear break point dividing the population into early (X61 type) and late (SL6 type) heading groups (data not shown). The dCAPS analysis of these F_3 plants showed that all early heading plants harbored the X61 allele in a homozygous state, whereas all late heading plants harbored the SL6 type allele either in a homozygous state or heterozygous state (Fig. 8). No recombinant was found. These results indicate that the *Se13* locus is surely *OsHY2*.

Discussion

In this study, we found that the extremely early heading of mutant line X61 is caused by the complete loss of photoperiodic response due to the single recessive mutant gene *se13* at the *Se13* locus. Subsequent linkage analysis revealed that the *Se13* locus is located between INDEL 3735_1 and INDEL3735_3 on chromosome 1. A database search for this region showed that AK101395 (=*OsHY2*) is a candidate for the *Se13* gene. As a result of sequence analysis of the *OsHY2* locus, X61 harbors a 1 bp insertion in exon 1 of *OsHY2*, which causes a frame-shift mutation producing a premature stop codon; therefore, we conclude that the *Se13* locus is identical to the *OsHY2* locus, and the *se13* gene is a loss of function allele at the *OsHY2* locus.

The *OsHY2* gene shares high homology with *Arabidopsis HY2* that encodes PΦB synthase protein and functions in the chromophore biosynthesis pathway. It is known that phytochrome action depends on its ability to photo-interconvert between the red-light-absorbing form (Pr) and the far-red-light-absorbing form (Pfr). This property is conferred by holo-phytochromes, which are produced by covalent bonding between PΦB and apo-phytochromes (e.g. *PHYA-E* in Arabidopsis, *PHYA-C* in rice) (Clack et al. 1994; Quail et al. 1995; Sharrock and Quail 1989; Basu et al. 2000; Takano et al. 2001, 2005). In Arabidopsis, the *HY1* gene encodes heme oxygenase, which converts heme protein to biliverdin (BV) IXα (Weller et al. 1997; Muramoto et al. 1999; Kochi et al. 2001), and the *HY2* gene encodes PΦB synthase, which reduces BV IXα to 3Z-PΦB (Terry et al. 1995). Therefore, genetic disruption of this linear tetrapyrrole synthesis offers a way to inactivate the entire phytochrome system, resulting in elimination of the plant's photoperiodic response (Terry 1997). In rice, Izawa et al. (2000) reported that the *se5* mutant is completely deficient in photoperiodic response and shows an extremely early heading even under long photoperiods. This is because *se5*, whose wild type allele is an ortholog of the Arabidopsis *HY1* gene, disturbs the conversion of heme to BV IXα in phytochrome chromophore biosynthesis. As described above, the *Se13* gene is a putative *OsHY2* gene that functions in the conversion of BV IXα to PΦB. In addition, the *se13* gene, like the *se5* gene, completely eliminates the photoperiodic response. It is therefore considered that the

complete deficiency of the photoperiodic response of X61 is caused by the loss of function of the *Se13* (=*OsHY2*) gene involved in phytochromobilin synthesis essential for photo-interconversion between Pr and Pfr.

It was found that the *Se13* locus interacts with two photoperiod sensitivity loci, *E1* and *Se1*, whose functional alleles do not function in coexistence with the non-functional allele *se13* in a homozygous state. In addition, although like the original variety Gimbozu, X61 harbors two major photoperiod sensitivity genes, *E1* and *Se1*, it looses photoperiod sensitivity completely. These results can be explained by the fact that the *Se13* locus is involved in phytochrome chromophore biosynthesis, which is essential for the functions of all genes involved in the genetic photoperiodic pathway to heading, such as the *E1* and *Se1* genes.

Rice was originally a short-day plant well-adapted to warm intermediate latitudes; therefore, commercial rice production had long been impractical in high latitudes, such as in Hokkaido (42–45°N), Japan and the Hei Long Jiang province (43–54°N), China, until early heading-time varieties with extremely weak photoperiod sensitivity and a short basic vegetative growth period were developed in the early twentieth century (Okumoto et al. 1996). Previous genetic analyses for heading time demonstrated that the varieties well-adapted to high latitudes all harbor *e1* at the *E1* locus, although most varieties of other regions, ranging from 20°N to 40°N, harbor *E1*, suggesting that *e1* is an essential gene of the high latitude varieties (Okumoto et al. 1996; Ichitani et al. 1998). On the other hand, in low latitudes, a recently established breeding program aims to produce varieties with a long basic vegetative growth period and weak photoperiod sensitivity. Such varieties will show almost constant vegetative growth periods under different photoperiods and thereby permit double or triple cropping in low latitudes. Therefore, weak- or non-photoperiod sensitivity varieties are required in both high and low latitudes. According to our previous studies, the non-functional alleles *e1* and *se1* markedly showed lower photoperiod sensitivity, but they never eliminated photoperiod sensitivity completely by themselves (Nishida et al. 2001; Tanisaka et al. 1992; Yamagata et al. 1986). In this sense, the mutant gene *se13*, which eliminates photoperiod sensitivity completely without the aid of other photoperiod insensitivity genes, will be helpful in the development of suitable varieties not only in high latitudes but also in low latitudes.

A lot of studies on photoperiodic control of flowering time have been performed using Arabidopsis. In rice, however, how photoperiods regulate heading time remains largely unclear. According to Tanisaka et al. (1992), there is a great variation in the photoperiod sensitivity among mutant lines, and several novel heading-time genes, including *se13* and *se5,* have been reported (Nishida et al. 2002; Saito et al. 2009; Yuan et al. 2009). Thus, induced heading-time mutants are quite useful for exploiting new genes related not only to photoperiod sensitivity but also to the basic vegetative growth period.

References

Basu D, Dehesh K, Schneider-Poetsch HJ, Harrington SE, McCouch SR, Quail PH (2000) Rice *PhyC* gene: structure, expression, map-position and evolution. Plant Mol Biol 44:27–42

Clack T, Mathews S, Sharrock RA (1994) The phytochrome apoprotein family in *Arabidopsis* is encoded by five genes: the sequences and expression of *phyD* and *phyE*. Plant Mol Biol 25:413–427

Hayama R, Coupland G (2004) The molecular basis of diversity in the photoperiodic flowering responses of Arabidopsis and rice. Plant Physiol 135:677–684

Ichitani K, Okumoto Y, Tanisaka T (1998) Genetic analyses of low photoperiod sensitivity of rice varieties from the northernmost regions of Japan. Plant Breed 117:543–547

Ichitani K, Inoue H, Nishida H, Okumoto Y, Tanisaka T (2002) Interactive effects of two heading-time loci, *Se1* and *Ef1*. on preflowering developmental phase in rice (*Oryza sativa* L.). Euphytica 126:227–234

Izawa T, Oikawa T, Tokutomi S, Okuno K, Shimamoto K (2000) Phytochromes confer the photoperiodic control of flowering in rice (a short-day plant). Plant J 22:391–399

Izawa T, Takahashi Y, Yano M (2003) Comparative biology comes into bloom: genomic and genetic comparison of flowering pathways in rice and Arabidopsis. Curr Opin Plant Biol 6:113–120

Kobayashi Y, Weigel D (2007) Move on up, it's time for change—mobile signals controlling photoperiod-dependent flowering. Genes Dev 21:2371–2384

Kochi T, Mukougawa K, Frankenberg N, Masuda M, Yokota A, Lagarias JC (2001) The Arabidopsis *Hy2* gene encodes phytochromobilin synthase, a ferredoxin-dependent biliverdin reductase. Plant Cell 13:425–436

Komari T (1989) Transformation of callus cultures of nine plant species mediated by Agrobacterium. Plant Sci 60:223–229

Koornneef M, Alonso-Blanco C, Peeters AJ, Soppe W (1998) Genetic control of flowering time in *Arabidopsis*. Annu Rev Plant Physiol Plant Mol Biol 49:345–370

Lin HZ, Liang W, Sasaki T, Yano M (2003) Fine mapping and characterization of quantitative trait loci *Hd4* and *Hd5* controlling heading date in rice. Breed Sci 53:51–59

Monden Y, Naito K, Okumoto Y, Saito H, Oki N, Tsukiyama T, Ideta O, Nakazaki T, Wessler SR, Tanisaka T (2009) High potential of a transposon *mPing* as a marker system in *japonica* x *japonica* cross in rice. DNA Res 16:131–140

Muramoto T, Kochi T, Yokota A, Hwang I, Goodman HM (1999) The Arabidopsis photomorphogenic mutant *hy1* is deficient in phytochrome chromophore biosynthesis as a result of a mutation in a plastid heme oxygenase. Plant Cell 11:335–348

Nishida H, Okumoto Y, Nakagawa H, Ichitani K, Inoue H, Tanisaka T (2001) Analysis of tester lines for rice (*Oryza sativa* L.) Heading-time genes using reciprocal photoperiodic transfer treatments. Ann Bot 88:527–536

Nishida H, Inoue H, Okumoto Y, Tanisaka T (2002) A novel gene *ef1-h* conferring an extremely long basic vegetative growth period in rice. Crop Sci 42:348–354

Okumoto Y, Ichitani K, Inoue H, Tanisaka T (1996) Photoperiod insensitivity gene essential to the varieties grown in the northern limit region of paddy rice (*Oryza sativa* L.) cultivation. Euphytica 92:63–66

Poonyarit M, Mackill DJ, Vergara VS (1989) Genetics of photoperiod sensitivity and critical day length in rice. Crop Sci 29:647–652

Quail PH, Boylan MT, Parks BM, Short TW, Xu Y, Wagner D (1995) Phytochromes: photo sensor perception and signal transduction. Science 268:675–680

Reeves PH, Coupland G (2001) Analysis of flowering time control in *Arabidopsis* by comparison of double and triple mutants. Plant Physiol 126:1085–1091

Saito H, Yuan Q, Okumoto Y, Doi K, Yoshimura A, Inoue H, Teraishi M, Tsukiyama T, Tanisaka T (2009) Multiple alleles at *Early flowering 1* locus making variation in the basic vegetative growth period in rice (*Oryza sativa* L.). Theor Appl Genet 119:315–323

Sano Y (1992) Genetic comparisons of chromosome 6 between wild and cultivated rice. Jpn J Breed 42:561–572

Sharrock RA, Quail PH (1989) Novel phytochrome sequences in *Arabidopsis thaliana*: structure, evolution and differential expression of a plant regulatory photoreceptor family. Genes Dev 3:1745–1757

Takano M, Kanegae H, Shinomura T, Miyao A, Hirochika H, Furuya M (2001) Isolation and characterization of rice phytochrome A mutants. Plant Cell 13:521–534

Takano M, Inagaki N, Xie X, Yuzurihara N, Hihara F, Ishizuka T, Yano M, Nishimura M, Miyao A, Hirochika H, Shinomura T (2005) Distinct and cooperative functions of phytochrome A, B and C in the control of de-etiolation and flowering in rice. Plant cell 17:3311–3325

Tanisaka T, Inoue H, Uozu S, Yamagata H (1992) Basic vegetative growth and photoperiod sensitivity of heading-time mutants. Jpn J Breed 42:657–668 (in Japanese with English summary)

Terry MT (1997) Phytochrome chromophore-deficient mutants. Plant Cell Environ 20:740–745

Terry MJ, McDowell MT, Lagarias JC (1995) (3Z)- and (3E)-phytochromobilin are intermediates in the biosynthesis of the phytochrome chromophore. J Biol Chem 270:11111–11118

Tsai KH (1995) Genetic analysis for heading time in wild rice strains. Jpn J Genet 70:555–562

Turck F, Fornara F, Coupland G (2008) Regulation and identity of florigen: FLOWERING LOCUS T moves center stage. Annu Rev Plant Biol 59:573–594

Valverde F, Mouradov A, Soppe W, Ravenscroft D, Samach A, Coupland G (2004) Photoreceptor regulation of CONSTANS protein in photoperiodic flowering. Science 303:1003–1006

Wang S, Basten CJ, Zeng ZB (2005) Windows QTL Cartographer 2.5. Department of Statistics, North Carolina State University, Raleigh, NC

Weller JL, Terry MJ, Reid JB, Kendrick RE (1997) The phytochrome-deficient pcd2 mutant of pea is unable to convert biliverdin IXα to 3Z-phytochromobilin. Plant J 11:1177–1186

Xue W, Xing Y, Weng X, Zhao Y, Tang W, Wang L, Zhou H, Yu S, Xu C, Li X, Zhang Q (2008) Natural variation in *Ghd7* is an important regulator of heading date and yield potential in rice. Nat Genet 40:761–767

Yamagata H, Okumoto Y, Tanisaka T (1986) Analysis of genes controlling heading time in Japanese rice. In "Rice genetics". IRRI, Manila, Philippines, pp 351–359

Yano M, Ebitani T (2002) Development of a series of chromosome segment substitution lines and their utilization in the genetic analysis of quantitative traits in rice. NIAS Annual Report, pp 27–28

Yano M, Harushima Y, Nagamura Y, Kurata N, Minobe Y, Sasaki T (1997) Identification of quantitative trait loci controlling heading date in rice using a high-density linkage map. Theor Appl Genet 95:1025–1032

Yano M, Katayose Y, Ashikari M, Yamanouchi U, Monna L, Fuse T, Baba T, Yamamoto T, Umehara Y, Nagamura Y, Sasaki T (2000) *Hd1*, a major photoperiod sensitivity quantitative trait locus in rice, is closely related to the *Arabidopsis* flowering time gene *CONSTANS*. Plant Cell 12:2473–2484

Yanovsky MJ, Kay SA (2002) Molecular basis of seasonal time measurement in *Arabidopsis*. Nature 419:308–312

Yokoo M, Okuno K (1993) Genetic analysis of earliness mutations induced in the rice cultivar Norin 8. Jpn J Breed 43:1–11

Yokoo M, Kikuchi F, Nakane A, Fujimaki H (1980) Genetical analysis of heading time by aid of close linkage with blast resistance in rice. Bull Natl Inst Agric Sci D31:95–126 (in Japanese with English summary)

Yuan Q, Saito H, Okumoto Y, Inoue H, Nishida H, Tsukiyama T, Teraishi M, Tanisaka T (2009) Identification of a novel gene ef7 conferring an extremely long basic vegetative growth phase in rice. Theor Appl Genet 119:675–684