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Regulatory mechanism of *OsPRR37* for heading date in rice

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ABSTRACT

Heading date and photoperiod sensitivity are fundamental traits that determine rice adaptation to a wide range of geographic environments. By quantitative trait locus (QTL) mapping and candidate gene analysis using whole genome re-sequencing, we found that *Oryza sativa Pseudo-Response Regulator37* (*OsPRR37*; hereafter *PRR37*) is responsible for the *Early Heading7-2* (*EH7-2*)/*Heading date2* (*Hd2*) QTL which was identified from a cross of late-heading rice 'Milyang23 (M23)' and early-heading rice 'H143'. H143 contains a missense mutation of an invariantly conserved amino acid in the CCT (CONSTANS, CO-like, and TOC1) domain of PRR37 protein. In the world rice collection, different types of nonfunctional *PRR37* alleles were found in many European and Asian rice cultivars. Notably, the *japonica* varieties harboring nonfunctional alleles of both *Ghd7/Hd4* and *PRR37/Hd2* flower extremely early under natural long-day conditions, and are adapted to the northernmost regions of rice cultivation, up to 53^{o} N

latitude. Genetic analysis revealed that the effects of *PRR37* and *Ghd7* alleles on heading date are additive, and *PRR37* down-regulates *Hd3a* expression to suppress flowering under long-day conditions. Our results demonstrate that natural variations in *PRR37/Hd2* and *Ghd7/Hd4* have contributed to the expansion of rice cultivation to temperate and cooler regions.

Keywords: rice; heading date; quantitative trait locus; natural variation; *Ghd7*; *OsPRR37*.

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CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATION

PRR	PSEUDO-RESPONSE REGULATOR
HIF	Heterogeneous inbred family
RIL	Recombinant inbred line
NIL	Near isogenic line
QTL	Quantitative trait loci
Hd	Heading date
LD	Long-day
SD	Short-day
DTH	Days to heading
ZT	Zeitgeber time

INTRODUCTION

Improvement of grain productivity in cereal crops requires seasonal and regional adaptation to different photoperiod environments at different latitudes. For example, in rice (*Oryza sativa*), environmental factors such as temperature, day length and cropping period affect the geographical adaptation of rice cultivars, and the regulation of flowering time (or heading date) is one of the key determinants for this adaptation (Izawa, 2007). Although rice is a facultative short-day (SD) plant whose flowering is accelerated under SD conditions (<10-h light/day) and is delayed under long-day (LD) conditions (>14-h light/day) (Izawa, 2007; Tsuji et al., 2008), current rice varieties are cultivated worldwide in a wide range of latitudes from 53^{o} N to 40^{o} S. Particularly at the northern extremes of rice cultivation, including Europe, northern Japan, northeastern provinces of China and the far-eastern parts of Russia (40-53^{o} N), elite rice cultivars flowers extremely early with very weak or no photoperiod sensitivity, which makes rice production possible under natural long day (NLD) conditions in a short summer period (Fujino and Sekiguchi, 2005; Wei et al., 2008). For global adaptation of rice to broad latitudes, diverse heading date and photoperiod sensitivity traits have been acquired by intensive artificial selection from the natural variants in rice germplasm (Luh, 1980; De Datta, 1981; Khush, 1997).

Photoperiod sensitivity is a major determinant for the transition from vegetative to reproductive phase in cereal crop plants, and is regulated by the interaction between endogenous circadian rhythms and exogenous daylengths, which varies at different geographical latitudes. Like the regulatory mechanisms of flowering time in *Arabidopsis* (*Arabidopsis thaliana*), rice heading date is a complex trait that is controlled by several independent genetic pathways. Many Hd-QTLs (*Hd1-Hd3a, Hd3b-Hd17*)

were found in the crosses of *japonica* and *indica* cultivars with distinct heading dates and photoperiod sensitivities (Yano et al., 2001; Uga et al., 2007; Monna et al., 2002). 'Nona Bokra', one of the *indica* rice cultivars used for the cross, exhibits extremely late heading with very strong photoperiod sensitivity, which is due to accumulation of additive effects of many Hd-QTLs (Uga et al., 2007).

Among the Hd-QTLs, several natural variants for Hd-QTL genes have been identified to date; these are *Se5* (Izawa et al., 2000), *Hd1* (Yano et al., 2000), *Hd6* (Takahashi et al., 2001), *Hd3a* (Kojima et al., 2002), *Ehd1/Hd14* (Doi et al., 2004), *Ghd7/Hd4* (Xue et al., 2008), *Hd5/DTH8/Ghd8* (Wei et al., 2010; Yan et al., 2010; Fujino et al., 2012), and *Ef7*/*OsELF3/Hd17* (Matsubara et al., 2012; Saito et al., 2012; Zhao et al., 2012). Molecular analysis revealed that diverse combinations of natural variation in *Hd1* alleles, two types of *Hd3a* promoter sequences and differential expression levels of *Ehd1* were found in the core collection of rice cultivars, leading to the diversity in flowering time (Takahashi et al., 2009). In addition, natural variation in *Ghd7/Hd4* contributes to rice cultivation in temperate and cooler regions (Xue et al., 2008). Fujino et al. (2012) recently reported that a loss-of-function *Hd5* allele is common in the rice population of Hokkaido, the northern limit of rice cultivation in Japan, and is useful for early-heading rice breeding programs. By several molecular genetics approaches, additional regulators of heading date have also been identified, such as *OsGI* (Hayama et al., 2003), *Ehd2/OsID1/RID1* (Matsubara et al., 2008; Park et al., 2008; Wu et al., 2008), *Ehd3* (Matsubara et al., 2011), *EL1* (Dai and Xue, 2010), *RFT1* (Komiya et al., 2008), *OsCO3* (Kim et al., 2008), *OsMADS50* and *OsMADS56* (Lee et al., 2004; Ryu et al., 2009), *OsMADS51* (Kim et al., 2007), *OsEF3* (Fu et al., 2009), *OsLFL1* (Peng et al., 2007), *OsVIL2* (Yang et al., 2012), and *DTH2* (Wu et al., 2013).

Extensive studies on the signaling mechanisms for photoperiod-dependent promotion or repression of flowering revealed that rice has both

conserved *Hd1* and unique *Ehd1* pathways to control the expression of the floral integrators *Hd3a* and *RFT1*, which act as rice florigens. *RFT1*, a rice ortholog of *Arabidopsis FT,* is up-regulated by *Ehd1* to promote flowering mainly in LD conditions (Komiya et al., 2009). *Ghd7* rarely affects flowering time in SD conditions, but strongly delays flowering by down-regulating *Ehd1* expression in LD conditions. *OsId1/RID1/Ehd2* acts as a master switch for flowering promotion by up-regulating *Ehd1* expression (Matsubara et al., 2008; Park et al., 2008; Wu et al., 2008), and *Ehd3* down-regulates *Ghd7* expression to up-regulate *Ehd1* expression in LD conditions (Matsubara et al., 2011). These indicate that rice heading date is controlled by SD activation, LD suppression, or LD activation pathways according to latitude-dependent daylengths (Izawa, 2007; Komiya et al., 2009).

Two-component system also known as histidine to aspartate phosphorelay is found in prokaryotes as a signal transduction mechanism. Common two-component system consists of sensor histidine kinase (HK) as a phosphor-donor and response regulator (RR) as a phospho-accepting receiver (Mizuno, 1998). HK can phosphorylate its related RR in response to specific stimulus, and the phospho-RR operates as a molecular switch especially at the transcription level (Mizuno and Nakamichi, 2005). This kind of system also exists in higher plant, *Arabidopsis*. ETR1 ethylene receptor is a well-known HK in *Arabidopsis* and AHKs (Arabodopsis HKs; AHK2, AHK3, and AHK4/CRE1) are serve as plant hormone, cytokinin, sensors (Chand and Shockey, 1999; Inoue et al., 2001; Suzuki et al., 2001; Ueguchi et al., 2001; Yamada et al., 2001).

RR-like proteins were found in Arabidopsis genome sequencing data. Their major differences between RR and RR-like proteins are phospho-accepting amino acid residue (Makino et al., 2000). RR-like proteins have non-phospho-accepting glutamate residue instead of phospho-accepting aspartate residue in the RR domain.

Pseudo-Response Regulators (*PRRs*) have been reported to be

important circadian-clock components in *Arabidopsis* (Alabadi et al., 2001; Kaczorowski and Quail, 2003; Yamamoto et al., 2003; Farre and Kay, 2007; Nakamichi et al., 2007; Ito et al., 2009). The five *Arabidopsis PRR* genes, *PRR1/TOC1*, *PRR3*, *PRR5*, *PRR7*, and *PRR9*, are expressed from dawn to dusk with different peak times in the order: *PRR9*, *PRR7*, *PRR5*, *PRR3*, and *PRR1* (Matsushika et al., 2000). Among them, *PRR9*, *PRR7*, and *PRR5* down-regulate the expression of *CCA1* and *LHY* through direct binding to their promoters during daytime in the feedback loop of the circadian clock (Nakamichi et al., 2010).

Rice also has a *PRR* gene family, including *OsPRR1/OsTOC1*, *OsPRR37*, *OsPRR59*, *OsPRR73*, and *OsPRR95* (Murakami et al., 2005; Murakami et al., 2007). *OsPRR37* is closely linked to *Hd2*, one of the major-effect Hd-QTLs controlling photoperiod sensitivity in rice (Murakami et al., 2005). The genes homologous to *OsPRR37*, such as *Ppd-H1* in barley, *Ppd-D1* in wheat, and *SbPRR37* in sorghum, have been well-studied about the functions in photoperiod sensitivity and the regulation of flowering-time genes (Turner et al., 2005; Beales et al., 2007; Murphy et al., 2011). Winter barleys usually sown in fall and went through low-temperature during winter. The low-temperature period is called vernalization that needs for emerging of ears in spring. This kind of flowering pattern is found in wild ancestor of barley, *Hordeum spontaneum*. On the other hand, spring barleys show weak or strong response to LDs and don't need to vernalization requirement. Mutated *ppd-H1* allele reduced photoperiod sensitivity through affects the expression of *HvFT* (Turner et al., 2005). *Ppd-D1a* on chromosome 2D, deleted 2kb upstream of coding region of *Ppd-D1* allele in hexaploid wheat, reduces photoperiod sensitivity (Beales et al., 2007). Its mutation changes wheat from a LD to a day-neutral plant, providing adaptation and cultivation to wide range of environments. Sorghum is a SD and a tropical plant that shows late flowering in LDs because of its photoperiod sensitivity (Murphy et al., 2011). *SbPRR37*, the major flowering regulator gene in sorghum, inhibits

heading in LD conditions. One of the natural variation allele of *SbPRR37* lost its repressing function in flowering, allowing sorghum to flower in temperate region through reduces photoperiod sensitivity. In particular, the natural variants of these homologous genes affect regional adaptability of cereal crop plants. In rice, however, the roles of *PRR37* in flowering pathways and regional adaptability of rice cultivars have remained elusive.

In this study, we performed Hd-QTL analysis using F_7 recombinant inbred lines (RILs) to identify the gene responsible for the *EH7-2*/*Hd2* QTL (Yoo et al., 2007). Through map-based cloning and whole genome re-sequencing, we found that *PRR37* is responsible for the *EH7-2/Hd2* QTL. The T-DNA mutant *prr37*-knockout (*prr37*-KO) plants flowered earlier than the parent rice Dongjin under LD conditions, due to up-regulation of *Hd3a* expression, indicating that *PRR37* mainly acts as a suppressor of LD-dependent flowering. By genotyping *PRR37* and *Ghd7* in the world rice collection, we demonstrate that natural variation in *PRR37* and *Ghd7* has contributed to *japonica* rice adaptation to growth in the northernmost regions of rice cultivation as well as double cropping of an *indica* variety in low-latitude regions by reducing days to heading (DTH) and photoperiod sensitivity.

MATERIALS AND METHODS

Plant materials and growth conditions

An extremely early flowering *japonica* rice 'H143' developed in Hokkaido, Japan (42-45^{o} N latitude) and the middle-late flowering rice 'Milyang23 (M23)' in Republic of Korea (36-37^{o} N latitude) were crossed to identify early Hd-QTLs in H143. M23 is a Korean Tongil-type rice cultivar (cv) that was bred from an *indica/japonica* hybridization and has a genetic makeup close to *indica* (>90%). A T-DNA knockout mutant of *PRR37* (LOC_Os07g49460; PFG_1B-02503.L; http://signal.salk.edu/cgi-bin/RiceGE) (Jeon et al., 2000) derived from the Korean late-flowering *japonica* rice cv 'Dongjin (DJ)' was used for the genetic validation of the *EH7-2* QTL for early flowering. The 264 F_7 recombinant inbred lines (RILs) and $F_{7:8}$ heterogeneous inbred family-near isogenic lines (HNILs) derived from F_7 RILs were used to test allele effects of *PRR37* and *Ghd7* as previously reported (Tuinstra et al., 1997).

Seeds were sown in the seed beds in the greenhouse on April 20 and transplanted to the paddy field on May 20. Plants were grown under natural long-day (NLD) conditions since the mean daylengths in Suwon, Republic of Korea (37^{o} N latitude) are 13.5 h in mid-April, 14.2 h in May, 14.7 h in June, 14.3 h in July and 13.6 h in August. The rice cultivation followed the normal agricultural practice in the paddy field of Seoul National University Experiment Farm. Plants were also grown in the growth chambers under SD (10-h light, 30^{o}C / 14-h dark, 24^{o}C) or LD conditions (14.5-h light, 30^{o}C / 9.5-h dark, 24^{o}C) with 60% relative humidity. The light source was light-emitting diodes with mixed red, blue, and white lights, and photon flux density was 500 μmol m^{-2} s^{-1}. Heading dates were recorded from sowing to the appearance of the first panicle of about 1 cm in length.

Whole genome re-sequencing

Genomic DNA was extracted from the leaf blades of 1-month-old rice plants using the NucleoSpin Plant II kit (Macherey-Nagel, Germany) according to the manufacturer's instructions. Whole genome re-sequencing was carried out using the Illumina Genome Analyzer IIx, and Illumina Pipeline 1.4 software was used as a sequencing platform.

Hd-QTL mapping

Genetic distances between the PCR-based markers on chromosome 7 were determined by Mapmaker 3.0 software using the Kosambi function (Kosambi, 1943; Lander et al., 1987). Q-gene 4.2.3 software was used for the Single Marker Regression (**Table 1**) using 264 F_7 RILs (Joehanes and Nelson, 2008). PCR-based markers including simple sequence repeat (SSR), single nucleotide polymorphism (SNP), and sequence-tagged site (STS) markers for high-resolution mapping were selected from previously reported molecular marker lists (Gramene) or newly developed by comparing the genomic or EST sequences of *indica* and *japonica* rice (**Table 2**).

Table 1. Hd-QTLs detected in the F_7 recombinant inbred lines (RILs) under natural long-day conditions in Suwon, Korea (37° N latitude) in 2008.

Nearest marker	Chr	LOD[a]	Additive effect[b]	PVE[c] (%)	QTL
RM5436	7	9.55	-4.02	16.1	*EH7-1/Hd4*[d]
RM22181	7	14.45	-4.91	24.1	*EH7-2/Hd2*[d]

All genetic parameters were calculated by means of composite interval mapping (CIM) using Q-gene ver. 4.2.3 (Joehanes and Nelson, 2008).

[a] Log-likelihood value.

[b] Additive effect of the Milyang23 allele on days to heading.

[c] Percent of phenotype variance explained by each QTL.

[d] Previously identified QTLs corresponding to the QTLs in this study based on their physical positions.

Table 2. Primers used in this study

Primer name	Forward (5' → 3')	Reverse (5' → 3')
RM427[a]	tcactagctctgccctgacc	tgatgagagttggttgcgag
RM481	tagctagccgattgaatggc	ctccacctcctatgttgttg
RM6574	aacctcgaattccttgggag	ttcgactccaaggagtgctc
S7042.6-1[b]	aatgcaaccggacttgagag	tatcatgcctggcgatacaa
S7047.7-1	tcgaattgtcaatcagataacg	tggttccatcctttgactcc
RM5436	tgagctgcacaagacagacaagc	accatttgaacaggatggactgg
RM21344	ggatgttgttctaacccgtcagg	cgaactcaacagactacccataccc
S7049.7-1-1	tgtcacactgctacgctatga	tttaccgagctgtgcctctt
S7050.0-1	tacacgaacgaacgacaagg	cgctgatttgggtaggtctc
S7053.4	cgaaactttgggacgaaatg	cgtccaccattcactgtcac
RM7110	ggcgatctctgtgtttattg	attaaccggttgagatggtg
S7067.0	tgagttcgtcgcacactgat	atgagttcatggtcgcacaa
RM346	cgagagagcccataactacg	acaagacgacgaggagggac
S7080.5	tcttccgtccatttctgctt	tcatcggcagtcaatttcag
S7088.7	cttcagtgcactcagccatc	tttttgctgcggtgattatg
RM234	acagtatccaaggccctgg	cacgtgagacaaagacggag
RM5455	ctcggcctgactagtcgatc	tgatggcgatcctgtgatag
S70101.8	tccaagcttgctttctttcc	tcggtacacacctcctgtga
S70103.4-1	agcatggatccttcatccaa	actccgatttttgcacttcg
RM3555	tggaagtttcctggcgatag	tggttggactgaaaagtccc
S70114.5	gttatcgtgtgggctttcgt	tctgttgctgcagctgaact
RM420	ggacagaatgtgaagacagtcg	actaatccaccaacgcatcc
RM22181	attctgggactggaggctcttgagg	tcgcctccatccatgtgattcc
RM22183	ctgtgctgtggtgatagatagc	gcacaaggaactgtgaattagc
RM22188	tttggaggctttctcgctttcg	gtcgaggatgaggaaggcatcg
SNP49480-1	agctcaaaaccatgtctctttg	tcagtgctgactgtaactccc
SNP49480-2	agctcaaaaccatgtctctttc	tcagtgctgactgtaactccc
*PRR37-0a*_H143	ccagagcagaaagaggcc	ccaaagccatcgcgtag
*PRR37-1*_M23	taccagagcagaaagaggct	ccaaagccatcgcgtag
PRR37-0b	aatgacatgggttccactac	tgctgcattgttagccactt

*PRR37-0c*_dCAPS_ KpnI	ggataggatcggtttctttctgatg	ctgctgttgttggtcttgcac
*PRR37-0d*_KS	cattttcaggtgcggtact	taggtaggtcatctgtccgc
PRR37-0d _WT	cattttcaggtgcggtacc	taggtaggtcatctgtccgc
Ghd7-0a	ttatccgttcatgtcgatgg	ttgccgaagaactggaacta
Ghd7-1	ttatccgttcatgtcgatgg	tgccgaagaactggaactc
OsGI-qPCR	atcgttctgcaggccgaga	tcaccaatgcttctgggctat
Hd1-qPCR	tcagcaacagcatatctttctcatca	tctggaatttggcatatctatcacc
Ehd1-qPCR[c]	gttgccagtcatctgcagaa	ggatgtggatcatgagacat
RFT1-qPCR[c]	tgacctagattcaaagtctaatcctt	tgccggccatgtcaaattaataac
PRR37-qPCR	gcaaagagggctcagcctgg	tcccattctgcccattgcttc
Hd3a-qPCR[c]	cttcaacaccaaggacttcgc	tagtgagcatgcagcagatcg
UBQ5-qPCR[d]	accacttcgaccgccactact	acgcctaagcctgctggtt

[a] The RM marker information was obtained from Gramene website (www.gramene.org).

[b] The STS marker information was provided by Koh, H. J. (Crop Molecular Breeding Lab. in Seoul National University).

[c] The primer information for *Ehd1*-qPCR, *RFT1*-qPCR and *Hd3a*-qPCR was obtained from Wu *et al*. (2008).

[d] *UBQ5*-qPCR marker information was obtained from Jain *et al*. (2006).

Genetic complementation test

The *Arabidopsis* wild-type and *prr7-3* mutant lines (Columbia-0 ecotype) were used in this study. All transgenic lines were generated by *Agrobacterium tumefaciens* transformation using a floral dip method (Zhang et al., 2006). Each *PRR37*(M23) and *PRR37*(H143) coding sequence was cloned by RT-PCR and inserted into the pMDC32 vector (Curtis and Grossniklaus, 2003). Transgenic *Arabidopsis* plants were selected on the plates with 3 mM MES buffer (pH5.8), 0.5X Murashige and Skoog medium, 15 mg/l hygromycin and 0.8% phytoagar.

RNA extraction, reverse transcription and quantitative real-time PCR

For reverse transcription and quantitative real-time PCR (RT-qPCR) or RT-PCR, total RNA was extracted from the leaves using the Easy-Spin Plant RNA Extraction Kit (iNtRON Biotechnology, Korea). Two μg of total RNA was used to synthesize first-strand cDNAs using M-MLV reverse transcriptase (Promega). Each RT reaction was diluted to 100 μl volume with water. For RT-qPCR, 2 μl of cDNA, 2 μl of 0.5 μM primers (**Table 2**), and 10 μl of 2X LightCycler 480 SYBR Green I Master Mix (Roche Applied Science) were used in 20 μl total reaction volume. Expression levels of each gene were

measured by the relative quantification method using a LightCycler 480 Real-Time PCR System (Roche Applied Science).

RESULTS

Identification of two major Hd-QTL genes conferring extremely early flowering under NLD conditions in the *japonica* rice H143

The *japonica* rice cultivated in the northern-limit regions (40-53^{o} N) flowers extremely early under NLD conditions, owing to natural variation in the genes controlling heading date and photoperiod sensitivity (Fujino and Sekiguchi, 2005; Xue et al., 2008). To identify the major Hd-QTLs for early flowering under NLD, we crossed two rice varieties, the extremely early-flowering *japonica* rice 'H143' (74 DTH) and the middle-late flowering Tongil-type rice 'Milyang23 (M23)' (116 DTH) (**Figure 1A** and **1B**). Based on Hd-QTL analysis with the resulting F_2 plants, we previously reported two major Hd-QTLs, *EH7-1* and *EH7-2* (Yoo et al., 2007), which are closely linked to *Hd4* and *Hd2*, respectively (Yano et al., 1997, 2001), on chromosome 7. *Hd2* and *Hd4*, among the major QTLs controlling heading date and photoperiod sensitivity in rice, were previously identified in the F_2 population generated by crossing a line that is nearly insensitive to photoperiod, *indica* cv. 'Kasalath (KS)', and a photoperiod-sensitive *japonica* cv. 'Nipponbare

(NB)' (Yano et al., 1997). For gene identification of *EH7-1/Hd4* and *EH7-2/Hd2* QTLs, we advanced the F_2 population to 264 F_7 recombinant inbred lines (RILs) by single seed descent (**Figure 2**). In the 264 RILs, DTH values were normally distributed from 72 to 126 under NLD conditions (**Figure 1C**).

Hd-QTL analysis using the F_7 RILs revealed that the Hd-QTLs *EH7-1* and *EH7-2* contribute to early flowering in H143 under NLD (**Table 1**). Among these two Hd-QTLs, we found that the *EH7-1* region includes *Ghd7*, which encodes a plant-specific CCT domain protein (**Figure 3A**). Null mutations in *Ghd7* reduce grain production and plant height, and promote early flowering under LD conditions (Xue et al., 2008). Sequence analysis revealed that H143 contains a nonfunctional *japonica*-type *Ghd7-0a* allele, leading to a premature termination of translation in exon 1, but M23 has a functional *indica*-type *Ghd7-1* allele (Xue et al., 2008), indicating that *Ghd7* is the gene underlying the *EH7-1* QTL. Thus, we next focused on identification of the gene responsible for the *EH7-2* QTL on chromosome 7 by analyzing candidate genes with next-generation sequencing and heterogeneous inbred family-near isogenic lines (HNIL) lines (Tuinstra et al., 1997) (**Figure 2**).

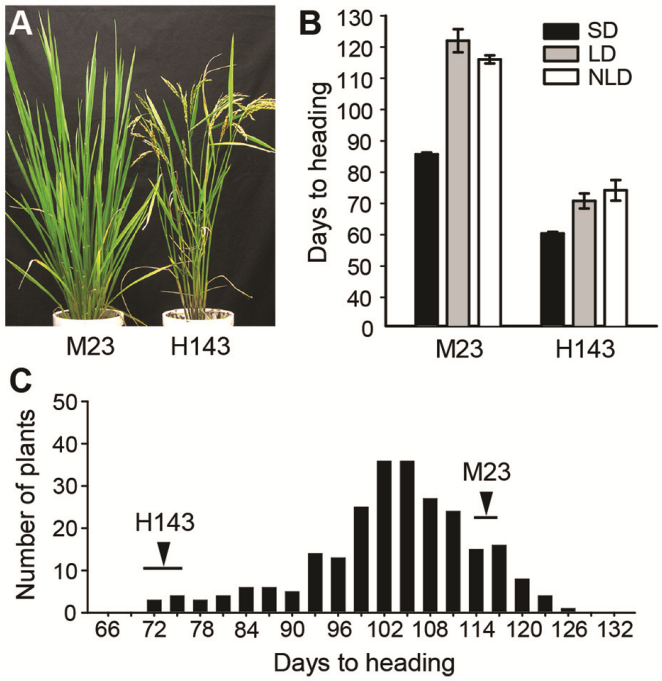


Figure 1. Days to heading of the parental rice cultivars M23 and H143, and F_7 recombinant inbred lines (RILs).

(A) Early-flowering phenotype of H143. Photo was taken after flowering of H143 under natural long-day (NLD) conditions. M23, Milyang23.

(B) H143 flowered earlier than M23, about 25 days under short-day (SD; 10-h light/day), 52 days under long-day (LD; 14.5 day/day) conditions, and 42 days under NLD conditions. Means and standard deviations were obtained from 40 plants of M23 and H143 for each daylength condition.

(C) Heading-date distribution of 264 F_7 RILs derived from the cross of M23 and H143. Means and standard deviations of heading dates in the parents, M23 and H143, are indicated as inverted triangles on the horizontal lines, which represent the range of heading dates of the parents, respectively.

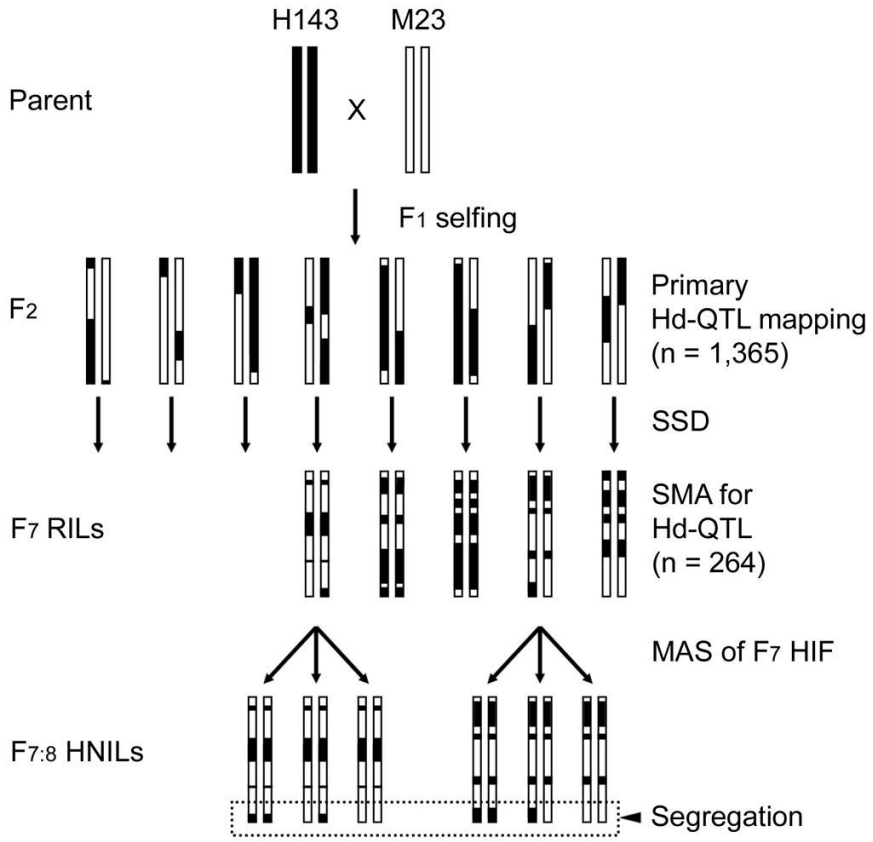


Figure 2. Schematic diagram of development of F_7 recombinant inbred lines (RILs), marker-assisted selection of F_7 heterogeneous inbred family (HIF) lines and analyses of heading dates in $F_{7:8}$ HIF-near isogenic lines (HNILs) from the cross of H143 and Milyang 23 (M23) in this study. Of 1,365 F_2 individuals derived from the cross of H143 x M23, 264 F_2 plants covering the range of heading dates in the F_2 population were selected for the development of F_7 RILs by single seed descent (SSD). By single marker analysis (SMA) with the advanced 264 F_7 RILs, the major Hd-QTLs that were previously identified in F_2 population (Yoo *et al*., 2007) were detected. Using the simple sequence repeat (SSR) marker RM22181, we selected two F_7 RILs that were heterozygous in the *EH7-2* locus and checked the segregation of heading dates in $F_{7:8}$ HNILs.

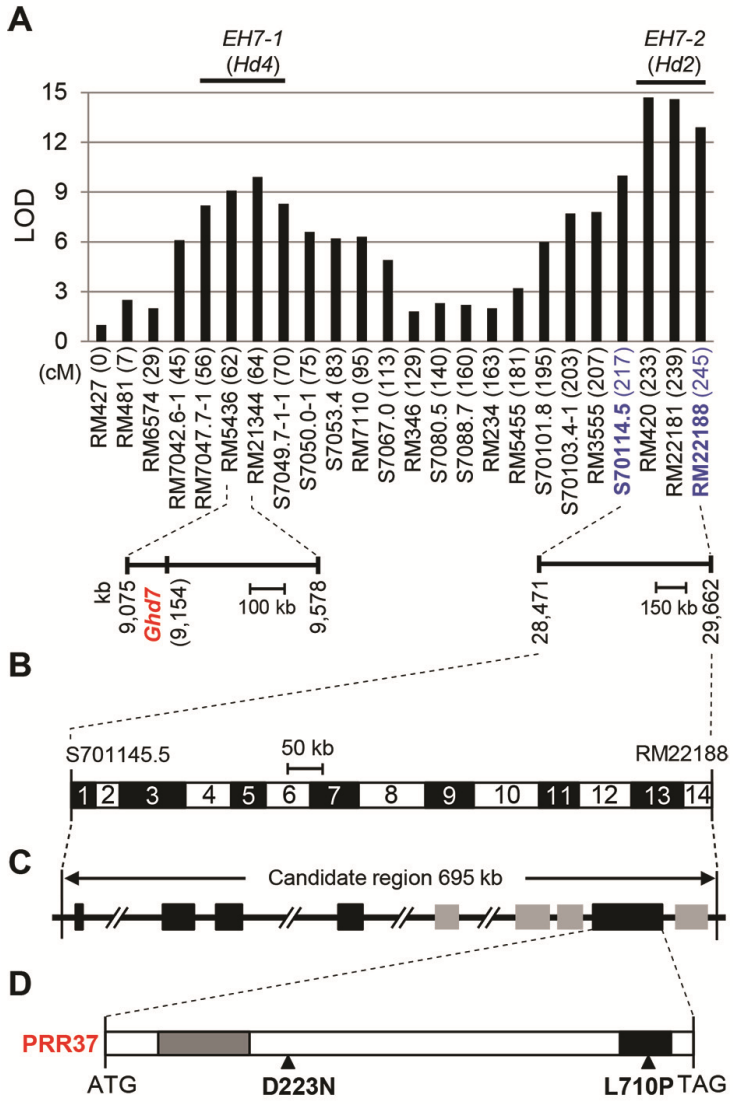


Figure 3. Heading-date quantitative trait locus (Hd-QTL) mapping and subsequent gene cloning of the *EH7-2/Hd2* QTL.

(A) Hd-QTL mapping was carried out using simple sequence repeat (SSR) and sequence tagged site (STS) markers on chromosome 7. Previously identified *Ghd7* is responsible for *EH7-1/Hd4* QTL detected between RM5436 and RM21344. *EH7-2/Hd2* QTL was detected between two markers, S70114.5 and RM22188.

(B) *EH7-2/Hd2* QTL region between S70114.5 and RM22188 markers has 14 bacterial artificial chromosome (BAC) clones. BAC clone accession numbers are as follows: 1, AP004300; 2, AP006269; 3, AP006268; 4, AP003817; 5, AP003757; 6, AP005243; 7, AP005099; 8, AP005167; 9, AP003818; 10, AP003813; 11, AP003816; 12, AP004316; 13, AP004333; 14, AP005199.
(C) Of 185 open reading frames (ORFs) in this region, 9 ORFs have amino acid substitutions. Sequences on chromosome 7 were read by Illumina Genome Analyzer IIx and base calling was performed using Illumina software Pipeline 1.4. Expressed genes and transposons are illustrated with black and gray boxes, respectively.
(D) Two unique nucleotide variations causing amino acid substitutions were found in the strongest candidate gene, *PRR37*. Gray and black boxes indicate the pseudoreceiver (PR) and CCT domains, respectively.

PRR37 is responsible for the *EH7-2*/*Hd2* QTL

To isolate candidate genes in the *EH7-2*/*Hd2* QTL region, we performed whole-genome re-sequencing of the two parental cultivars, M23 and H143 (see Methods). Based on the sequence information obtained from whole-genome re-sequencing, we compared the nucleotide sequences of the genomic fragments between S70114.5 and RM22188 markers in M23 and H143. In this candidate region, 185 predicted genes were identified by the Rice Functional Genomic Express (RiceGE) database (**Figure 3B**), and the sequences of 185 open reading frames (ORFs) were compared between M23 and H143; NB was used as a reference cultivar. Of 185 genes, 9 ORF sequences were exactly the same between M23 and NB, but different from H143 (**Figure 3C**). These 9 ORFs consist of five expressed and four retrotransposon genes (**Table 3**). Among five expressed genes, *Pseudo-Response Regulator 37* (*PRR37*), an ortholog of *Arabidopsis PRR7* (Murakami et al., 2005), was identified as a strong candidate gene. *PRR37* encodes 742 amino acids (aa) and contains an N-terminal pseudoreceiver (PR) domain (61-181 aa) and a C-terminal CCT domain (682-723 aa) (**Figure 3D**). Several nucleotide polymorphisms in the *PPR37* coding sequence have been reported between *indica*-type KS and *japonica*-type NB (Murakami et al., 2005) and we also found many nucleotide polymorphisms in the *PPR37* coding sequence that differed between M23

and H143 (**Figure 4**). The *indica*-type *PRR37-1* in M23 is predicted to be functional, but H143 has a natural variant of *japonica*-type *PRR37* (*PRR37-2a*) that contains four aa substitutions compared to the functional *japonica*-type *PRR37-2* in NB (Murakami et al., 2007) (**Figure 4**). Among these four aa differences in H143, two residues, aspartate to asparagine (D223N) and leucine to proline (L710P), are unique among the subspecies-specific *PRR37* alleles in rice. Of the two aa differences, L710P is predicted to disrupt PRR37 function in the regulation of heading date and photoperiod sensitivity in rice, because L710 aa is invariantly conserved in the CCT domains of PRR members in higher plants, whereas D223 was highly variable among species (**Figure 5**).

To determine the effect of the H143-type *PRR37* (*PRR37-2a*) allele, termed *PRR37*(H143) hereafter, on heading date, we examined the DTH phenotype of different RILs with different *PRR37* genotypes. To this end, we genotyped all 264 RILs and identified two F_7 RILs (#106 and #177) that were heterozygous *PRR37*(M23/H143) and homozygous *Ghd7*(M23). Sequencing of RT-PCR products for *Hd1* revealed that RIL#106 and RIL#177 have homozygous *hd1* (type 7, 4-bp deletion in M23) and *Hd1* (type 9 in H143) alleles, respectively (based on the *Hd1* allele information from Takahashi et al., 2009). We next confirmed that heading dates in the $F_{7:8}$ progenies derived from each of the two F_7 RILs segregated with a range from 98 to 129 DTH under NLD in the paddy field, although morphological phenotypes of the plants were almost homogeneous (data not shown). For

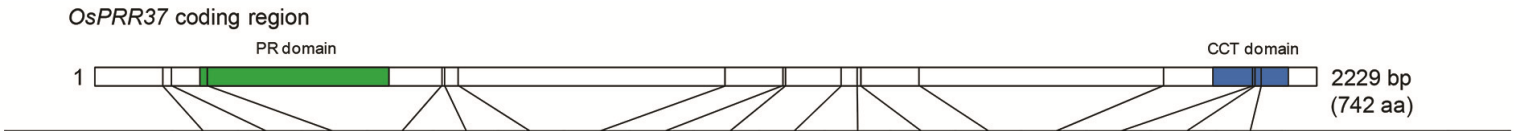
further verification, heading dates of the $F_{7:9}$ progenies produced from the $F_{7:8}$ plants harboring homozygous *PRR37*(M23) or *PRR37*(H143) alleles, termed $F_{7:9}$ HNILs such as HNIL(M23) and HNIL(H143), were examined under SD and LD conditions in the growth chamber as well as under NLD conditions in the paddy field. Under all three photoperiod conditions, HNIL(H143) plants flowered earlier than HNIL(M23) plants, which were observed in two different genetic lines RIL#106(*hd1*) and RIL#177(*Hd1*) (**Figure 6**). This strongly suggests that natural missense mutation (L710P) in the CCT domain of *PRR37*(H143) caused early flowering independent of *Hd1* activity, and photoperiod sensitivity (differences in DTH between SD and LD) was considerably reduced in HNIL(H143) compared to HNIL(M23) (**Figure 6**).

Murakami et al. (2007) demonstrated that long-period free-running rhythms, red light-dependent longer hypocotyl and late-flowering phenotypes in the *Arabidopsis prr7-3* mutants were genetically complemented by the rice *PRR37,* indicating that the rice *PRR37* is a true ortholog of *Arabidopsis PRR7*. Thus, we tested the function of *PRR37*(H143) by performing a genetic complementation test to determine whether the *Arabidopsis prr7-3* mutant can be complemented by *35S:PRR37*(M23) and *35S:PRR37*(H143) (see Methods). We found that several transgenic lines failed to complement the late-flowering phenotype of *prr7-3* mutants under LD conditions (**Figure 7**), strongly suggesting that *PRR37*(H143) is nonfunctional. To further validate the early-flowering effect of *prr37* on

heading date and photoperiod sensitivity, we examined DTH of the T-DNA insertion *prr37* knockout (*prr37*-KO) mutants in the late-flowering *japonica* rice 'Dongjin' (121 DTH) background (Jeon et al., 2000). The *prr37*-KO mutants showed reduced photoperiod sensitivity and flowered earlier than the Dongjin (DJ) rice under all three photoperiod conditions (**Figure 8**), reminiscent of the phenotype of HNIL(H143) plants (**Figure 6**). Taken together, these results indicate that the natural variant *PRR37*(H143) is nonfunctional, and *PRR37* plays important roles in increasing photoperiod sensitivity and also acts as a flowering suppressor in rice, especially in LD conditions.

Table 3. List of candidate genes for *EH7-2*/*Hd2* between S70114.5 and RM22188 markers on chromosome 7

No.	MSU ID	Gene product name	Amino acid substitution
1	Os07g48440	Conserved hypothetical protein	H176R
2	Os07g48570	Expressed protein	D400N
3	Os07g48630	Ethylene-insensitive 3-like protein	R110Q
4	Os07g49080	COBRA-like protein 7 precursor	R489G
5	Os07g49170	Retrotransposon	A84V
6	Os07g49430	Retrotransposon	T532A, V689A
7	Os07g49440	Retrotransposon	L60V, R534Q
8	Os07g49460	Response regulator receiver domain containing protein (*PRR37*)	D223N, L710P
9	Os07g49500	Retrotransposon	Q711R, D1234N, D1308E



	Nucleotide position	125	140	204	641	642	667	1147	1259	1266	1369	1385	1397	1515	1915	2110	2113	2129	Alleles		No. of Plants
	aa substitution	I42T	R47P	D67E	N214S	-	**D223N**	A383T	G420D	-	M457V	L462P	S466I	**F.S.**	D639N	**Y704H**	**Q705@**	**L710P**	***PRR37***	***Ghd7***	
indica	Milyang23	C	C	G	G	T	G	A	A	T	G	C	T	G	A	T	C	T	***PRR37-1***	***Ghd7-1***	4
	Nanxiongzaoyou	C	C	G	G	T	G	A	A	T	G	C	T	G	A	T	C	T		***Ghd7-0***	1
	Nanjing11	C	C	G	G	T	G	A	A	T	G	C	T	**8 bp del**	A	T	C	T	***PRR37-1a***	***Ghd7-1***	16
	Zhenshan97	C	C	G	G	T	G	A	A	T	G	C	T	**8 bp del**	A	T	C	T		***Ghd7-0***	1
	Wukezhan	C	C	G	G	T	G	A	A	T	G	C	G	G	A	**C**	C	T	***PRR37-1b***	***Ghd7-1***	6
	Kasalath	C	C	T	A	C	G	G	A	T	G	C	T	G	G	T	**T**	T	***PRR37-1c***		10
japonica	Nipponbare	T	G	T	A	T	G	G	G	C	A	T	G	G	G	T	C	T	***PRR37-2***	***Ghd7-2***	3
	Dongnong416	T	C	T	A	T	**A**	G	G	C	A	T	G	G	A	T	C	**C**	***PRR37-2a***		8
	H143	T	C	T	A	T	**A**	G	G	C	A	T	G	G	A	T	C	**C**		***Ghd7-0a***	15

Figure 4. Natural variation in nucleotide and amino acid (aa) sequences of *PRR37* in Asian rice varieties. Polymorphic nucleotides are marked in different colors. In particular, the variation and alleles causing loss-of-function types are colored in yellow. Cultivars are divided into two subspecies, *indica* and *japonica*, and the allele types of *PRR37* and *Ghd7*. The aa substitutions are indicated, as well as F.S. (frameshift), del (deletion), and @ (stop codon) changes.

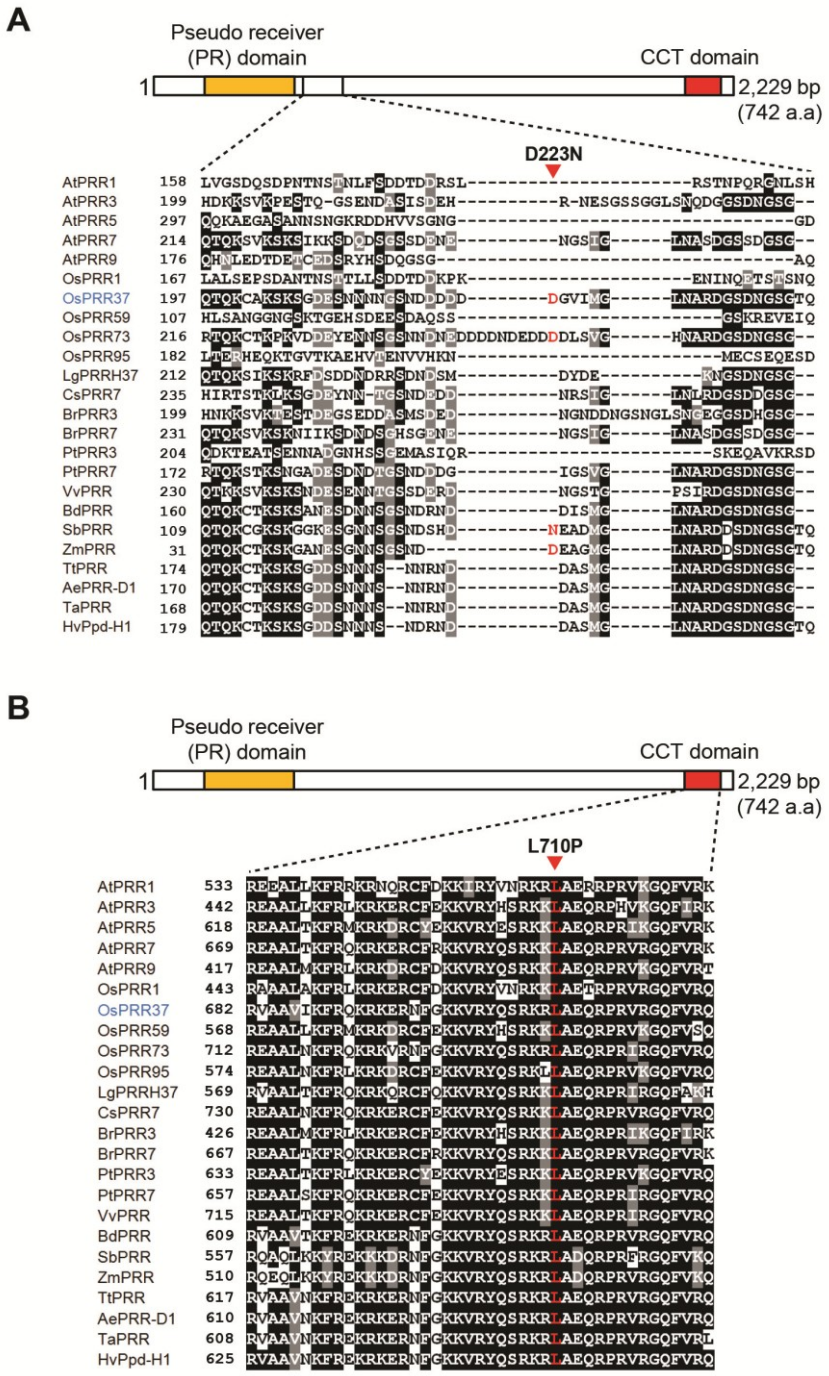


Figure 5. Amino acid (aa) sequence alignments of higher plant PRRs and two causative mutations in the coding region of PRR37 in H143. Amino acid

alignment of N-terminal regions (A) and CCT domains (B) of *PRR* genes in higher plants. Sequence accessions were obtained from the NCBI protein sequence database (http://www.ncbi.nlm.nih.gov/protein); AtPRR1 (*Arabidopsis thaliana*, AB041530), AtPRR3 (AB046956), AtPRR5 (AB046955), AtPRR7 (AB046954), AtPRR9 (AB046953), OsPRR1 (*Oryza sativa* [cv. Nipponbare], AB189038), OsPRR37 (AB189039), OsPRR59 (ABG22372), OsPRR73 (AB189040), OsPRR95 (AB189041), LgPRRH37 (*Lemna gibba*, AB243684), CsPRR7 (*Castanea sativa*, EF694004), BrPRR3 (*Brassica rapa*, GU219473), BrPRR7 (*Brassica rapa*, CO749922), PtPRR3 (*Populus trichocarpa*, XM_002321313), PtPRR7 (XM_002311088), TtPRR (*Triticum turgidum,* EU117149), AePRR-D1 (*Aegilops tauschii*, DQ885771), TaPRR (*Triticum aestivum*, DQ885766), HvPpd-H1 (*Hordeum vulgare* [cv. Igri], AY970701). Sequence accessions from Gramene are VvPRR (*Vitis vinifera*, GSVIVG00024444001), BdPRR (*Brachypodium distachyon*, BRADI1G16490), SbPRR (*Sorghum bicolor*, Sb06g014570), ZmPRR (*Zea mays*, GRMZM2G005732). Red inverted triangles represent highly variable 223th aspartic acid (A) and invariantly conserved 710th leucine (B).

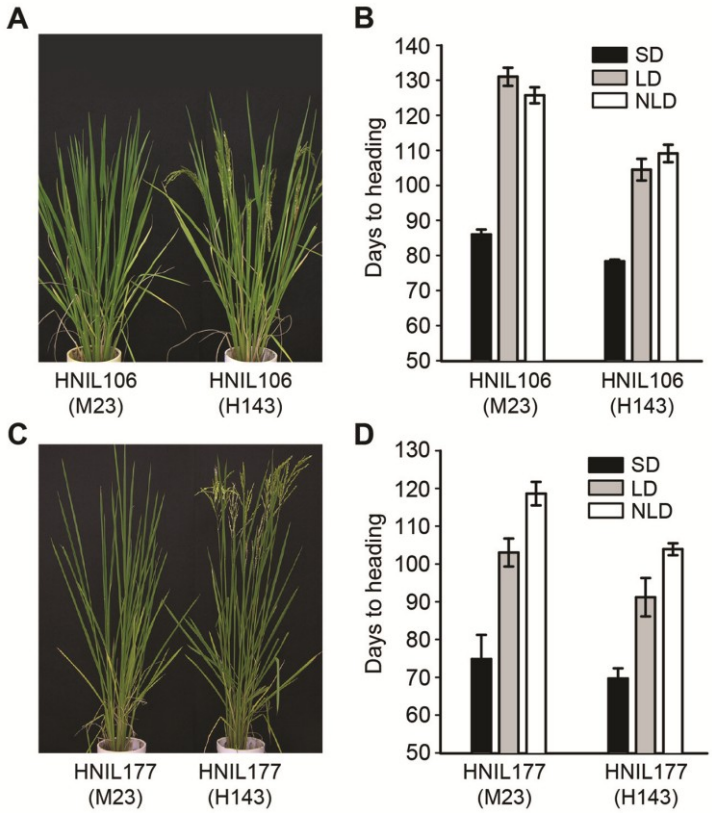


Figure 6. Days to heading (DTH) of two HNILs under different photoperiodic conditions.

(A) The heading phenotypes of HNIL106(M23) and HNIL106(H143) under natural long-day (NLD) conditions. Photo was taken after heading of HNIL106(H143) plant.

(B) DTH of HNIL106(M23) and HNIL106(H143) under short-day (SD), long-day (LD), and NLD conditions. HNIL106(H143) flowered earlier than HNIL106(M23) in all three conditions.

(C) The heading phenotypes of HNIL177(M23) (left) and HNIL177(H143) (right) under NLD conditions. Photo was taken after heading of HNIL177(H143) plant.

(D) HNIL177(H143) flowered earlier than HNIL177(WT) under SD, LD, and NLD conditions. Bars are mean values ± standard deviations.

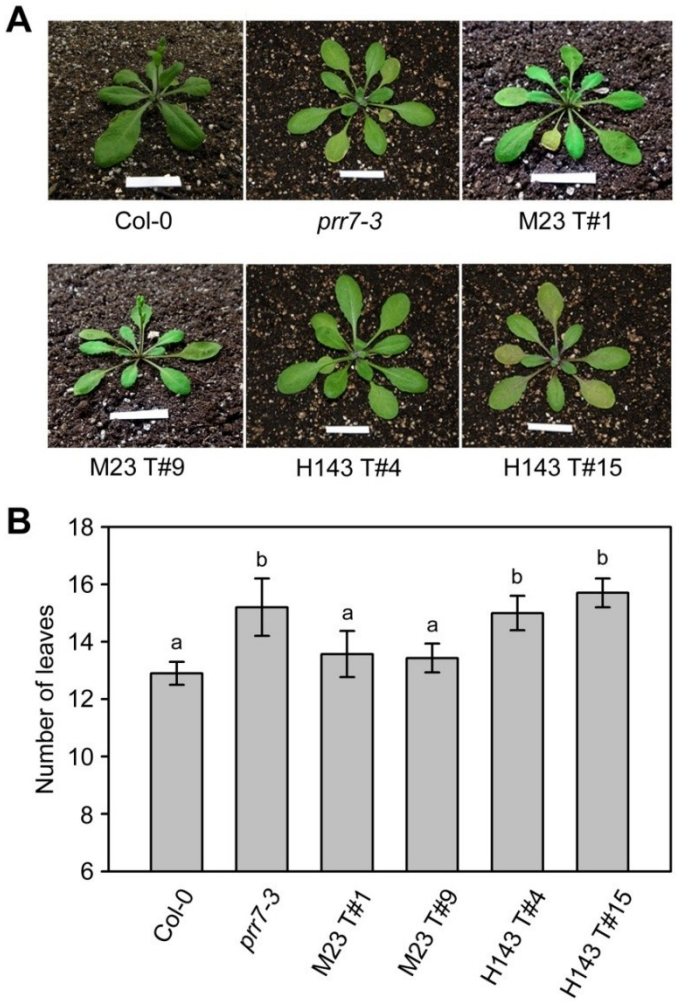


Figure 7. The expression of *PRR37*(H143) failed to complement the late-flowering phenotype of Arabidopsis *prr7-3* mutants in long-day (LD) condition. Plants grown under LD conditions (16-h light / 8-h dark) were used to record flowering time.

(A) Flowering phenotypes of WT (Col-0) plants, *prr7-3* mutants and four independent transgenic lines M23 T#1, M23 T#9, H143 T#4 and H143 T#5. M23 T#1, M23 T#9, H143 T#4 and H143 T#15 represent the *35S:PRR37*(M23)/*prr7-3* T#1, *35S:PRR37*(M23)/*prr7-3* T#9, *35S:PRR37*(H143)/*prr7-3* T#4 and *35S:PRR37*(H143)/*prr7-3* T#15

transgenic lines, respectively. Photos (A) were taken when WT plants were bolting. Scale bars, 2 cm.

(B) Leaf numbers were recorded after the primary inflorescence was bolted about 0.5 cm. More than 8 plants for each line were used for means and SDs. a and b on the bars indicate that they are significantly different at the 5% level according to the Duncan's multiple range test. This experiment was performed three times with similar results.

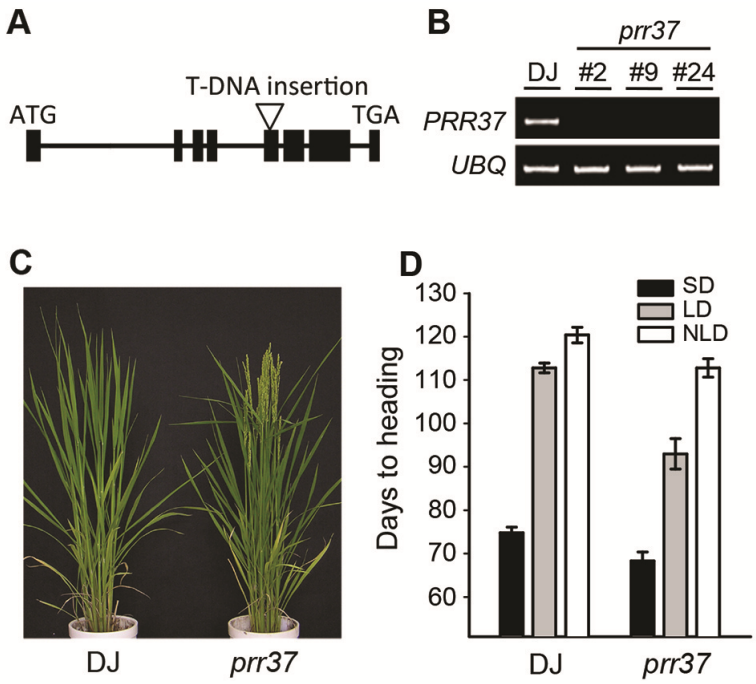


Figure 8. Early flowering of *prr37*-KO mutants under different photoperiodic conditions.

(A) Schematic diagram of the T-DNA insertion in the 5th exon of *PRR37* in the *japonica* rice 'Dongjin (DJ)'.

(B) Absence of *PRR37* transcripts in *prr37*-KO mutants derived from DJ. *PRR37* transcript was detected in only wild-type DJ. *Ubiquitin* (*UBQ*) was used as an internal control.

(C) Heading-date phenotypes of DJ and *prr37*-KO mutants grown under natural long-day (NLD) conditions in the paddy field. Photo was taken at 1 day after heading of *prr37*-KO mutants.

(D) *prr37*-KO mutants flowered earlier than DJ, approximately 7 days in short-day (SD), 20 days in long-day (LD) and 8 days in NLD conditions. Means and standard deviations were obtained from 20 plants (SD and LD) and 40 plants (NLD) of each genotype.

Natural variation in *PRR37* has contributed to rice cultivation to a wide range of latitudes

To evaluate the effect of mutations in *PRR37* on rice adaptation to a wide range of latitudes, including the northern-limit regions of rice cultivation (53^{o} N), we examined the genotypes of rice varieties that have been conventionally cultivated in Japan, China and India (**Figure 4, Table 4**). By sequence analysis of the *PRR37 c*oding region, we found various nonfunctional variants in *indica* rice cultivars, *PRR37-1a* (a frameshift mutation; 8-bp deletion) and *PRR37-1b* (Y704H) and *PRR37-1c* (premature stop in KS), and in *japonica* rice, *PRR37-2a* (L710P). Most of the amino acid changes outside of the PR and CCT domains in *PRR37* represent subspecies-specific variations that are not predicted to disrupt function. Since *EH7-1*/*Hd4* was identified as a major-effect QTL along with *EH7-2*/*Hd2* contributing to the photoperiod-insensitive early flowering phenotype in H143 (**Figure 3**), we also examined the genotypes of *Ghd7*/*Hd4* in the rice cultivars using STS and SNP markers (**Figure 4**).

The region of rice cultivation representing each allele was marked on the map of Asia (**Figure 9**), and the DTH of each cultivar was recorded in rice plants cultivated in Suwon (37^{o} N; ~14.5-h light/day) or Beijing (39^{o} N; ~15-h light/day) under NLD conditions (**Table 4**). *PRR37-2a* single or *PRR37-2a*/*Ghd7-0a* double mutations were found in the *japonica* rice

cultivated in the high-latitude regions of northeastern Asia (**Figure 9**). In most cases, the *japonica* cultivars carrying double mutations flowered earlier in Suwon than those containing a single mutation (**Table 4**). By contrast, the *indica* cultivars with a single mutation (*PRR37-1a*, *PRR37-1b* or *PRR37-1c*) were widely cultivated in the low-latitude regions of Asia and displayed a broad range of heading dates in Suwon, Korea (**Figure 9**). These results suggest that naturally occurring mutations in *PRR37* and *Ghd7* play an important role in rice adaptation from low to high latitudes, especially in the distribution of *japonica* rice cultivars to the northern-limit regions up to 53^{o} N.

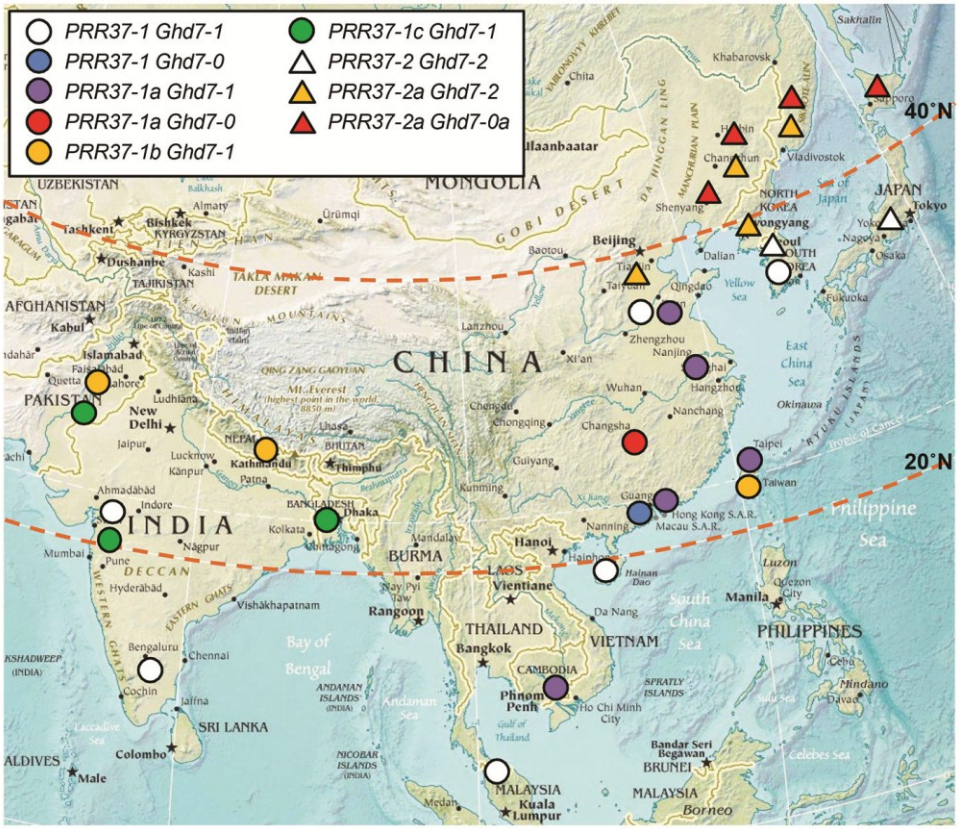
Table 4. Heading dates and allele types of *PRR37* and *Ghd7* in rice collection

Sub-species	Cultivar	HD[a]	*PRR37*	*Ghd7*	Origin	Latitude
indica	Miyang23	113(S)	*PRR37-1*	*Ghd7-1*	S. Korea	37
indica	Guichao 2[b]	112(S)	*PRR37-1*	*Ghd7-1*	Guangxi, China	22
indica	Menjiading	93(B)	*PRR37-1*	*Ghd7-1*	Hainan, China	19
indica	Pei'ai64S[b]	113(S)	*PRR37-1*	*Ghd7-1*	Hunan, China	28
indica	Nanxiongzaoyou	50(B)	*PRR37-1*	***Ghd7-0***	Guangzhou, China	23
indica	Nanjing 11[b]	105(S)	***PRR37-1a***	*Ghd7-1*	Jiangsu, China	32
indica	93-11	n.d	***PRR37-1a***	*Ghd7-1*	Jiangsu, China	32
indica	K4481-1-2[b]	86(S)	***PRR37-1a***	*Ghd7-1*	India	8-29
indica	Africa[b]	116(S)	***PRR37-1a***	*Ghd7-1*	India	8-29
indica	Ai-Nan-Tsao1[b]	91(S)	***PRR37-1a***	*Ghd7-1*	China	n.d
indica	British Honduras Creale[b]	108(S)	***PRR37-1a***	*Ghd7-1*	Honduras	13-16
indica	CH1039[b]	86(S)	***PRR37-1a***	*Ghd7-1*	India	8-29
indica	Chachme[b]	100(S)	***PRR37-1a***	*Ghd7-1*	Taiwan	22-25
indica	Cica 4[b]	118(S)	***PRR37-1a***	*Ghd7-1*	Cambodia	10-14
indica	Italica M-1[b]	79(S)	***PRR37-1a***	*Ghd7-1*	Russia	43-53
indica	J.K689[b]	99(S)	***PRR37-1a***	*Ghd7-1*	India	8-29
indica	Kam Baungan[b]	101(S)	***PRR37-1a***	*Ghd7-1*	Hong Kong	22
indica	Kwang-Lu-Ai 4[b]	84(S)	***PRR37-1a***	*Ghd7-1*	China	n.d
indica	Marichbeti[b]	96(S)	***PRR37-1a***	*Ghd7-1*	India	8-29
indica	MTU10[b]	111(S)	***PRR37-1a***	*Ghd7-1*	India	8-29
indica	RT1095-326[b]	105(S)	***PRR37-1a***	*Ghd7-1*	Senegal	13-16
indica	Zhenshan 97	63(B)	***PRR37-1a***	***Ghd7-0***	Jiangxi, China	28
indica	Wukezhan	106(B)	***PRR37-1b***	*Ghd7-1*	Fujian, China	26
indica	IRRI13382[b]	97(S)	***PRR37-1b***	*Ghd7-1*	Pakistan	24-34
indica	Kataktara[b]	101(S)	***PRR37-1b***	*Ghd7-1*	Pakistan	24-34
indica	Sokan Dhan[b]	101(S)	***PRR37-1b***	*Ghd7-1*	Nepal	27-30
indica	Zoeow Shani[b]	103(S)	***PRR37-1b***	*Ghd7-1*	Hungary	46-48
indica	Gogak Nanju[b]	99(S)	***PRR37-1b***	*Ghd7-1*	Taiwan	22-25
indica	Qimei	121(B)	***PRR37-1b***	*Ghd7-1*	Guangdong, China	23

Sub-species	Cultivar	HD[a]	*PRR37*	*Ghd7*	Origin	Latitude
indica	Kasalath	105(S)	***PRR37-1c***	*Ghd7-1*	India	8-29
indica	CTG1680[b]	92(S)	***PRR37-1c***	*Ghd7-1*	Bangladesh	22-26
indica	DE78[b]	97(S)	***PRR37-1c***	*Ghd7-1*	Pakistan	24-34
indica	DNJ142[b]	95(S)	***PRR37-1c***	*Ghd7-1*	Pakistan	24-34
indica	Dubor[b]	99(S)	***PRR37-1c***	*Ghd7-1*	Bangladesh	22-26
indica	IRRI8828[b]	111(S)	***PRR37-1c***	*Ghd7-1*	Pakistan	24-34
indica	Hashikalmi[b]	94(S)	***PRR37-1c***	*Ghd7-1*	Suriname	3
indica	Kataktara Da-2[b]	101(S)	***PRR37-1c***	*Ghd7-1*	Pakistan	24-34
indica	Nizer Sail[b]	90(S)	***PRR37-1c***	*Ghd7-1*	Bangladesh	22-26
indica	Pukhi[b]	98(S)	***PRR37-1c***	*Ghd7-1*	Pakistan	24-34
japonica	Nipponbare	116(S)	*PRR37-2*	*Ghd7-2*	Japan	35
japonica	Dongjin	116(S)	*PRR37-2*	*Ghd7-2*	S. Korea	37
japonica	Koshihikari	105(S)	*PRR37-2*	*Ghd7-2*	Japan	35
japonica	Kantori[b]	77(S)	*PRR37-2*	***Ghd7-0a***	Russia	43-53
japonica	Longjing 8[b]	76(S)	*PRR37-2*	***Ghd7-0a***	Heilongjiang, China	43-53
japonica	Xiangnianmi[b]	94(S)	*PRR37-2*	***Ghd7-0a***	Jilin, China	40-46
japonica	Songjing 3	n.d	***PRR37-2a***	*Ghd7-2*	Heilongjiang, China	43-53
japonica	Dongnong 416	n.d	***PRR37-2a***	*Ghd7-2*	Heilongjiang, China	43-53
japonica	Fengdao 12[p]	95(S)	***PRR37-2a***	*Ghd7-2*	Jilin, China	40-46
japonica	Solaris[b]	87(S)	***PRR37-2a***	*Ghd7-2*	Russia	43-53
japonica	Vonya 2[b]	96(S)	***PRR37-2a***	*Ghd7-2*	N. Korea	39
japonica	Italica Oobie[b]	77(S)	***PRR37-2a***	*Ghd7-2*	Russia	43-53
japonica	Bulgare[b]	98(S)	***PRR37-2a***	*Ghd7-2*	Hungary	46-48
japonica	K-3753 Italica (Alex)[b]	88(S)	***PRR37-2a***	*Ghd7-2*	Russia	43-53
japonica	H143[p,c]	78(S)	***PRR37-2a***	***Ghd7-0a***	Hokkaido, Japan	43-45
japonica	N11[c]	79(S)	***PRR37-2a***	***Ghd7-0a***	Hokkaido, Japan	43-45
japonica	H75[c]	79(S)	***PRR37-2a***	***Ghd7-0a***	Hokkaido, Japan	43-45
japonica	Iburiwase[d]	72(S)	***PRR37-2a***	***Ghd7-0a***	Hokkaido, Japan	43-45
japonica	Kitaibuki[d]	71(S)	***PRR37-2a***	***Ghd7-0a***	Hokkaido, Japan	43-45
japonica	Hayamasari[d]	73(S)	***PRR37-2a***	***Ghd7-0a***	Hokkaido, Japan	43-45

Sub-species	Cultivar	HD[a]	*PRR37*	*Ghd7*	Origin	Latitude
japonica	Hoshinoyume [d]	74(S)	***PRR37-2a***	***Ghd7-0a***	Hokkaido, Japan	43-45
japonica	Hejiang 19[b]	79(S)	***PRR37-2a***	***Ghd7-0a***	Heilongjiang, China	43-53
japonica	Mudanjiang 8 [b]	47(B)	***PRR37-2a***	***Ghd7-0a***	Heilongjiang, China	43-53
japonica	Yanjing 19 [p]	75(S)	***PRR37-2a***	***Ghd7-0a***	Jilin, China	40-46
japonica	Mudanjiang 19 [b]	81(S)	***PRR37-2a***	***Ghd7-0a***	Heilongjiang, China	43-53
japonica	Yan 304 [p]	81(S)	***PRR37-2a***	***Ghd7-0a***	Jilin, China	40-46
japonica	Hejiang 195 [b]	81(S)	***PRR37-2a***	***Ghd7-0a***	Heilongjiang, China	43-53
japonica	Horebare [b]	84(S)	***PRR37-2a***	***Ghd7-0a***	Japan	35
japonica	Yannong 1 [p]	76(S)	***PRR37-2a***	***Ghd7-0a***	Jilin, China	40-46

[a] Heading date (Hd) of each cultivar was recorded in Suwon, Korea (S, 37º N) or Beijing, China (B, 39º N). n.d : not determined.
[b] The seeds were provided from National Agrobiodiversity Center, Rural Development Administration, Korea.
[c] The seeds were provided from Plant Breeding Laboratory, Faculty of Agriculture, Hokkaido University, Japan.
[d] The seeds were provided from Hokkaido Prefectural Central Agriculture Experiment Station, Japan.



Source of the map: http://www.vidiani.com/?p=10784

Figure 9. Geographical distribution of rice varieties harboring different alleles of *PRR37* and *Ghd7* in Asia.

The *indica* and *japonica* varieties are marked by circles and triangles, respectively. Note that the *japonica* varieties harboring two nonfunctional alleles of *PRR37* (*PRR37-1a, PRR37-1b, PRR37-1c* and *PRR37-2a*) and *Ghd7* (*Ghd7-0* and *Ghd7-0a*) are largely cultivated in northern-limit regions of rice cultivation in Asia.

PRR37 delays heading date by repressing *Hd3a* under LD conditions

To establish the regulatory role of *PRR37* in photoperiodic flowering pathways, we examined the expression of key photoperiod regulators in *prr37*-KO mutants. The *prr37*-KO mutants and the wild-type rice 'Dongjin (DJ)' were grown under SD and LD conditions in the growth chambers and harvested at 50 and 70 days after germination, respectively. Reverse transcription and quantitative real-time PCR (RT-qPCR) analysis of whole leaves revealed that expression patterns and levels of *OsGI*, *Ghd7*, *Hd1*, *Ehd1*, and *RFT1* were not altered in *prr37*-KO mutants under LD conditions (**Figure 10A-E**). The expression level of *Hd3a*, however, was significantly up-regulated in *prr37*-KO mutants (**Figure 10F**). Under SD conditions, expression levels of *OsGI*, *Ghd7*, *Hd1*, *Ehd1*, *RFT1*, and *Hd3a* also did not show a significant difference between *prr37*-KO and WT plants under SD conditions (**Figure 11**). In rice, it has been shown that *Hd3a* is regulated by two upstream genes *Hd1* and *Ehd1* (Doi et al., 2004). In particular, *Hd3a* is up-regulated by *Hd1* under SD and down-regulated by *Hd1* under LD (Yano et al., 2000; Hayama et al., 2003). These results demonstrated that *PRR37* functions as a repressor of *Hd3a* expression independent of *Hd1*-*Hd3a* and *Ehd1*-*Hd3a* pathways.

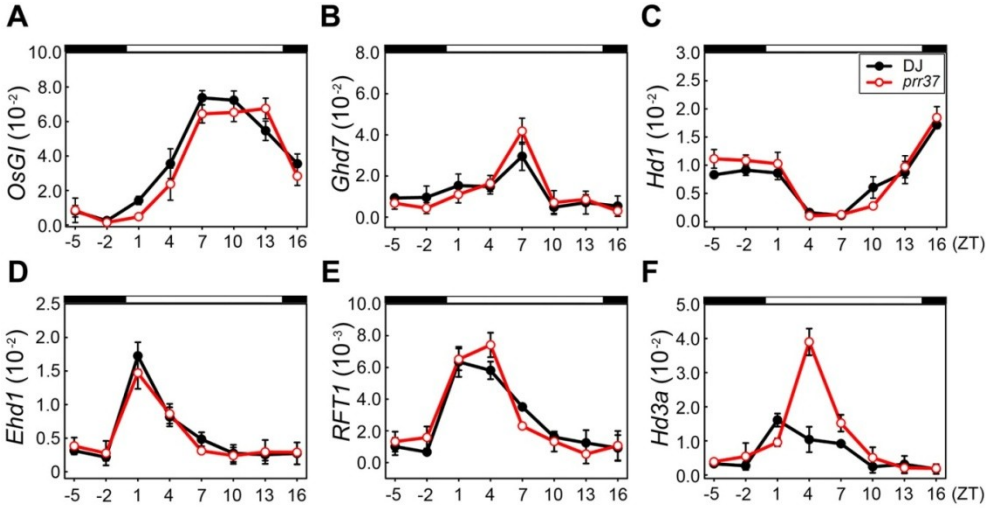


Figure 10. Transcript levels of flowering genes in Dongjin (DJ) and *prr37*-KO mutants under long-day (LD) conditions. Total RNA was extracted from the mature leaves of plants grown under LD conditions, at 70 days after seeding. Relative expression levels of *OsGI* (A), *Ghd7* (B), *Hd1* (C), *Ehd1* (D), *RFT1* (E), and *Hd3a* (F) were measured by RT-qPCR, and normalized to the transcript levels of *UBQ5* (LOC_01g22490). Note that only *Hd3a* expression increased in *prr37*-KO mutants. Solid black and empty red circles represent DJ and *prr37*-KO mutants, respectively. Means and standard deviations were obtained from three biological replicates. This experiment was replicated at least three times with similar results. ZT, zeitgeber time.

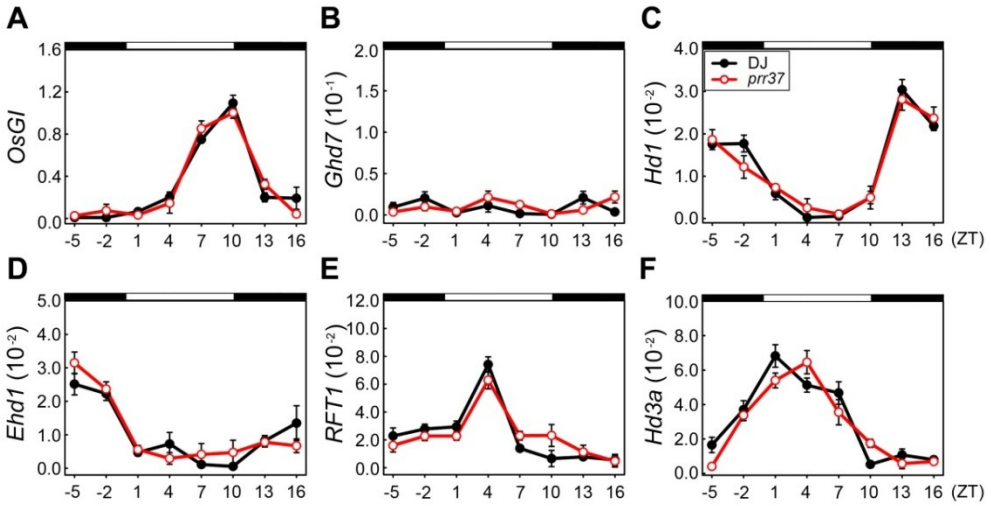


Figure 11. Transcript levels of flowering genes in Dongjin (DJ) and *prr37*-KO mutants under short-day (SD) conditions. Total RNA was extracted from the mature leaves of plants grown under SD conditions at 50 days after seeding. By RT-qPCR analysis, relative expression levels of *OsGI* (A), *Ghd7* (B), *Hd1* (C), *Ehd1* (D), *RFT1* (E), and *Hd3a* (F) were obtained by normalizing to the transcript levels of *UBQ5*. Note that no significant difference was observed in gene expression between DJ and *prr37*-KO mutants. Solid black and empty red circles represent DJ and *prr37*-KO mutants, respectively. Means and standard deviations were obtained from three biological replicates. This experiment was replicated at least four times with similar results. ZT, zeitgeber time.

Discussion

Natural variation in *PRR37* has contributed to rice cultivation to a wide range of latitudes

Several studies have reported that two major-effect Hd-QTLs, *Hd2*/*qDTH7-2* and *Hd4*/*qDTH7-1*, mainly contributed to rice cultivation in northern-limit regions by decreasing photoperiod sensitivity and DTH (Fujino & Sekiguchi 2005; Nonoue et al., 2008; Shibaya et al., 2011). *Ghd7*, which underlies the *Hd4*/*qDTH7-1* QTL, was identified by map-based cloning and the effect of *Ghd7* on heading date and photoperiod sensitivity was studied in different rice cultivars (Xue et al., 2008). The rice varieties cultivated in high-latitude regions (northeastern China) have a nonfunctional *Ghd7* allele resulting in an early-heading phenotype (Xue et al., 2008). By contrast, low-latitude (tropics and subtropics area) rice cultivars have a functional *Ghd7* allele and a late-heading phenotype. Natural variants of *PRR37,* which is responsible for *Hd2/qDTH7-2* QTL, have also been reported (Murakami et al., 2005). Several nucleotide polymorphisms in the coding sequences of *PPR37* were found between *indica*-type Kasalath (KS) and *japonica*-type Nipponbare (NB). Among them, a nonsense mutation (Gln-705-stop)

occurred at the CCT domain in KS (Murakami et al., 2005). Our coding sequence analysis of *PRR37* in this study revealed that a missense mutation (Leu-710-Pro) in the invariably conserved Leu-710 residue of the CCT domain may have caused loss-of-function of PRR37 in H143. Further genotyping of *PRR37* and *Ghd7* in the world rice collection revealed that the *japonica* cultivars harboring nonfunctional *PRR37-2a* and functional *Ghd7-2* alleles were distributed across northern Asia, suggesting that *PRR37* plays a major role in rice adaptation to high-latitude regions by reducing photoperiod sensitivity and causing early flowering. In addition, double mutations in *PRR37/Hd2* and *Ghd7/Hd4* were commonly detected in many rice varieties that flower early in LD conditions. The *japonica* cultivars with these two mutations were distributed mostly in Hokkaido, Heilongjiang and Jilin (**Table 4**, **Figure 9**), which are in the northern-limit regions of paddy rice, with more than 15-h mean daylength during the short growing season (Fujino, 2003). However, the cultivars with natural variants of *indica*-type *PRR37* displayed a broad range of heading dates in Suwon, Korea (**Table 4**) and were widely distributed in the low-latitude regions of Asia. Notably, the *indica* rice variety 'Zhenshan 97' harboring *PRR37-1a*/*Ghd7-0* double mutations was found in a low-latitude region. This might be due to the artificial domestication of *indica* rice cultivars for the system of two-rice cropping in the central and southern China, in which the life cycle is much shorter (Xue et al., 2008).

Recently, it was reported that a natural variant of *Hd5* also promoted

early heading and contributed to rice distribution in high-latitude regions along with nonfunctional *hd2* and *hd4* alleles (Fujino et al., 2012). Consistent with this, we also found that extremely early heading 'Kitaibuki' and 'Hayamasari' cultivars have nonfunctional alleles of *Ghd7-0a* and *PRR37-2a* (**Table 4**). These findings suggested that rice domestication in the northernmost regions has been achieved by natural variants in two major-effect QTL genes, *PRR37/Hd2* and *Ghd7/Hd4*, as well as variants in other minor-effect QTL genes, such as *Hd5* and *Hd6*, all of which act mainly as regulators of increasing photoperiod sensitivity or repressors of LD-dependent flowering in rice.

Epistatic interactions between *PRR37/Hd2* and other Hd-QTLs

Epistatic interactions of *Hd2* with several Hd-QTLs have been identified by genetic analysis in various genetic backgrounds. For example, *Ghd7*/*Hd4* and *Hd2* showed epistatic interaction, and this interaction was observed only with the functional *Hd4* allele, but not the nonfunctional *hd4* allele (Fujino and Sekiguchi, 2005; Shibaya et al., 2011). By contrast, Lin et al. (2003) reported that *Hd4* has no epistatic interaction with *Hd2*. Here, we found no epistatic relationship between *Ghd7* and *PRR37* in our genetic analysis (**Figure 12**). We analyzed allele effects of *PRR37* and *Ghd7* using the four genotype classes of $F_{7:8}$ HNIL segregants. The rice plants carrying nonfunctional *PRR37-2a* and *Ghd7-0a* showed extremely early heading, and the allele effects of *PRR37-2a* and *Ghd7-0a* on DTH were additive

under NLD conditions (**Figure 12**). In addition, expression levels of *Ghd7* were not altered in *prr37*-KO mutants compared to the parent rice 'Dongjin (DJ)' under both LD and SD conditions (**Figure 10B, Figure 11B**). These results strongly suggest that *Ghd7* and *PRR37* act independently in LD-dependent flowering pathways in rice.

An epistatic interaction between *Hd2* and *Hd6* has also been reported (Yammamoto et al., 2000). The *indica* rice KS has nonfunctional *hd2* and functional *Hd6* alleles, and the *japonica* rice NB has functional *Hd2* and nonfunctional *hd6* alleles. DTH of the NILs harboring the *hd2*(KS) allele were not affected by the *Hd6* or *hd6* genotypes in LD, suggesting that *Hd2* is epistatic to *Hd6* in the LD-dependent flowering pathways in rice. Ogiso et al. (2010) reported that *Hd6*, encoding CK2α, inhibits flowering under LD conditions and *Hd1* is required for the flowering-inhibitory function of *Hd6*, possibly through CK2-mediated phosphorylation. They showed that *Hd1* and *Ghd7*, acting as repressors in the LD-dependent flowering pathway, are not phosphorylated *in vitro* by Hd6, suggesting that these proteins are not direct targets of Hd6. In this respect, it can be considered that Hd6 may phosphorylate other floral inhibitor(s), possibly PRR37/Hd2, to activate under LD conditions because *Hd2* is epistatic to *Hd6*. Further genetic analysis using HNILs segregating for alleles of various heading-date genes, and *in vivo* and *in vitro* kinase assays need to be conducted to elucidate the biochemical mechanism of *Hd2*-mediated flowering repression in LD.

From the QTL study of rice heading, *Hd2*, *Ghd7*, and *Hd5* were reported to be candidate modulators of downstream flowering genes such as *Ehd1* and *Hd3a* under LD conditions (Ebana et al., 2011). However, we found that only *Hd3a* was up-regulated in *prr37*-KO mutants without alteration in the expression of genes upstream of *Hd3a*, including *Ehd1* and *Hd1* (**Figure 10**). This indicates that in rice, *PRR37* suppresses the expression of *Hd3a* independent of expression of *Ehd1* and *Hd1* (**Figure 13**). By contrast, sorghum *SbPRR37*, an ortholog of *Arabidopsis PRR7*, delays flowering by

repressing *SbEhd1* expression in LD (Murphy et al., 2011). In addition, barley *Ppd-H1*, an ortholog of rice *PRR37,* functions as a major determinant of heading date by up-regulating *HvFT* expression, because the peak and expression levels of the barley CO-like genes, *HvCO1* and *HvCO2*, were shifted and reduced in *ppd-h1* mutants under LD conditions, respectively (Turner et al., 2005). However, recent studies showed that the barley *Ppd-H1* allele affects *HvFT* expression independent of *HvCO1* mRNA (Campoli et al., 2012; Faure et al., 2012), indicating that *Ppd-H1* may affect *HvFT* expression by controlling the stability or activity of HvCO1 protein (Campoli et al., 2012). Further *in vitro* and *in vivo* binding assays of PRR37 to the *cis*-elements of *Hd3a* promoter regions as well as protein-protein interaction assays between PRR37 and Ehd1 or Hd1 are necessary to elucidate the biochemical mechanism of PRR37-mediated *Hd3a* repression under LD conditions.

It was reported that *Hd2* is not only photoperiod-related but also a temperature-related QTL (Nakagawa et al., 2005). Temperature is another environmental factor affecting rice heading; under low temperature in LD conditions, increased expression of *Ghd7* delays heading through down-regulation of *Ehd1* expression (Song et al., 2012). In addition, temperature-sensitive *japonica* rice cultivars are distributed in the low-latitude regions in China (Wei et al., 2009). In *Arabidopsis*, *PRR7* and *PRR9* play important roles in temperature compensation mechanisms, and *prr7 prr9* double mutants fail to maintain a constant free-running period at low or high temperature (Salome et al., 2010). Although *Hd2* was reported as a major-effect Hd-QTL responding to altered temperature, the *PRR37*/*Hd2* function in this mechanism is still unknown. The relationship of temperature sensitivity and domestication of rice at high latitudes to natural variation in *PRR37* also remains to be elucidated. Taken together, our results demonstrate that *PRR37* plays a crucial role as a floral repressor in LD, and natural variation of *PRR37* enabled rice to be domesticated and cultivated in

high-latitude regions. This finding provides a breeding resource to develop elite cultivars for stable rice production in the northernmost regions of rice cultivation.

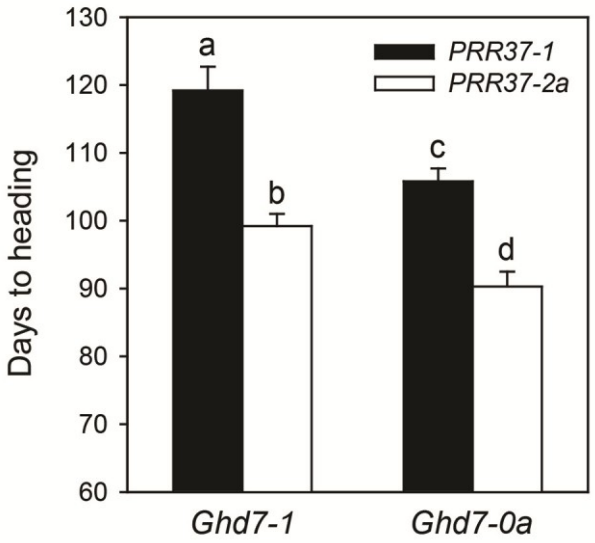


Figure 12. *PRR37* and *Ghd7* do not function in the same flowering-related pathway. Differences in days to heading in four genotype classes of F_8 HNIL segregants under natural long-day (NLD) conditions. Two populations of HNILs derived from F_7 RIL132 (homozygous *PRR37-2a* and heterozygous *Ghd7-1*/*Ghd7-0a* alleles) and F_7 RIL257 (homozygous *PRR37-1* and heterozygous *Ghd7-1*/*Ghd7-0a* alleles) were used to test allele effects of *PRR37* and *Ghd7*. Genotype of each plant was checked using SNP markers (**Table 2**). Forty plants were used in each genotype for the measurement of heading date. Bars are mean values ± standard deviations. The P value was calculated by using a 1-way analysis of variance (ANOVA).

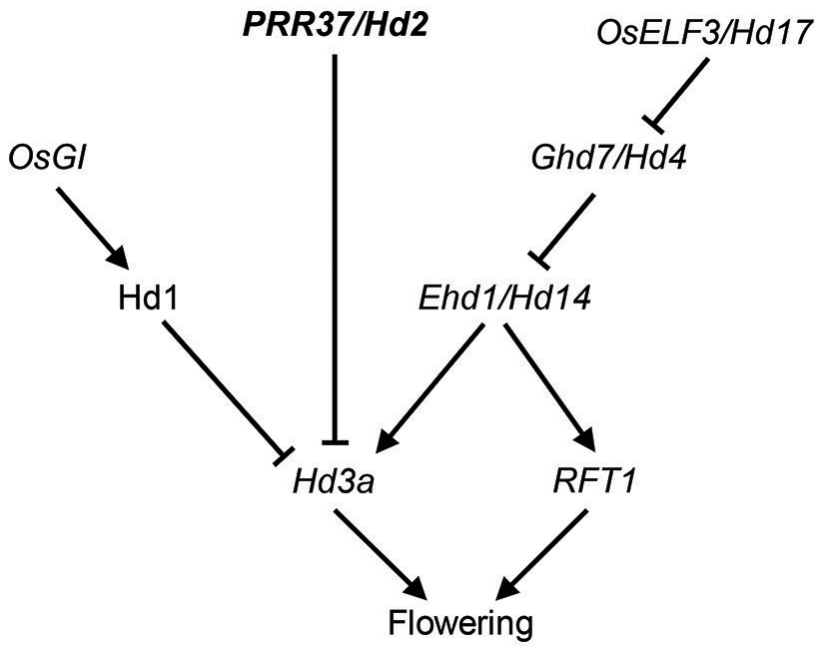


Figure 13. Schematic model depicting the role of *PRR37* in the rice flowering pathway under natural long-day conditions. *PRR37* delays rice flowering via down-regulation of *Hd3a* expression without alteration of expression of genes upstream of *Hd3a* in the pathway.

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초 록

벼 *OsPRR37* 유전자의 개화기 조절 기전 연구

일본 북해도 원산인 극조생종 벼 H143 유전자원과 한국 중남부에서 재배되는 중만생종 벼인 밀양 23 호를 교배하여 얻은 RIL 집단을 이용하여 *Pseudo-Response Regulator 37* (*OsPRR37*) 유전자를 지도기초분리 후 기능 연구를 통해 *OsPRR37* 유전자가 벼의 출수 조절에 관여함을 밝힌 연구이다. 벼의 출수에 관여한다고 알려져 있었던 *Heading date 2* (*Hd2*) QTL 이 *OsPRR37* 임을 밝혔고 *OsPRR37* 기능이 정상인 밀양 23 호의 allele 을 가진 집단과 *OsPRR37* 기능이 소실된 H143 의 allele 을 가진 집단을 비교한 결과 자연적 장일조건인 포장에서 H143 allele 을 가진 집단이 밀양 23 호의 allele 을 가진 집단보다 조기개화 하였다. 즉 *OsPRR37* 이 장일조건에서 개화를 지연하는 개화억제자로서 광주기 민감성 조절에 관여함을 알 수 있었다. 장일조건에서 *OsPRR37* 의 밀양 23 호 allele 은 개화를 촉진하는 florigen 으로 알려진 *Hd3a* 의 발현을 억제하여 개화를 억제하는 기능을 하는 것으로 나타났다. H143 의 *OsPRR37* 과 같은 자연변이는 이러한 개화 억제 기능을 상실했으며 일본과 중국 고위도 지역에서 재배되는 벼 품종에서도 H143 allele 의 자연변이를 발견하였고 이러한 품종들은

고위도 지역의 벼 재배 기간동안 장일조건에서도 조기 개화하는 특징을 보였다. 이는 자연선택과 인간에 의한 인위적 선발에 의해 벼의 원산지인 저위도 열대지역에서부터 고위도 한냉지역으로 순화되었음을 증명하는 자료가 된다.