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ORIGINAL ARTICLE

Assessment of the effect of ten heading time genes on reproductive transition and yield components in rice using a CRISPR/Cas9 system

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Abstract

Key message **We demonstrated the effect of heading time genes on reproductive transition and yield components under an identical genetic background using CRISPR/Cas9 gene-editing technology, and we propose that the elite allele will provide a new breeding strategy for rice breeding in high-latitude regions.**

Abstract Heading date is a factor closely associated with grain yield in rice (*Oryza sativa* L). In recent decades, a number of genes responsible for heading time have been identified, the variation of which contributes to the expansion of the rice cultivation area. However, it is difficult to compare the phenotypic effects of these genes due to the different genetic backgrounds. In this study, we generated 14 heading time mutants using CRISPR/Cas9 gene-editing technology and marker-assisted selection with a *japonica* Sasanishiki wild-type (WT) genetic background. Photoperiod sensitivity, the relationship between days to heading (DTH), and yield components of mutants were investigated. We found that the yield increases with increases in DTH, but eventually plateaus at maximum and then began to decrease, whereas the biomass continued to increase. The mutants exerted distinctly different effects on DTH and yield components. The convergent double mutants had severe yield reduction compared with single mutants, even with a DTH that was similar to that of single mutants. We also found that an elite mutant of *se14* achieved a yield equal to that of the WT, but with heading occurring 10 days earlier. A sequence analysis of 72 cultivars collected from the *japonica* cultivated zone shows that elite *se14* mutants have not been applied to rice breeding. Our study demonstrates the effect of heading time genes on reproductive transition and yield components under an identical genetic background. These results may provide new insights into rice breeding using heading time mutants.

Introduction

Major advancements in crop domestication often come from the naturally occurring genetic variation in the universal florigen pathway (Park et al. 2014). In rice (*Oryza sativa* L.), heading (or flowering) time is closely associated with grain yield. The appropriate heading time guarantees successful reproduction and balances vegetative and reproductive growth. The genetic regulation of heading time is a gene network of an antagonistic regulation of accelerated pathways under short-day (SD) conditions and repressed pathways under long-day (LD) conditions (Komiya et al. 2009). The *Early heading date 1* (*Ehd1*) gene functions as a signal integrator under both SD and LD conditions (Doi et al. 2004). *Ehd1* enhances the expression of *Heading date 3a* (*Hd3a*) and *Rice FT-like 1* (*RFT1*), two florigen-like genes in rice (Takahashi et al. 2009), and while *edh1* mutant delays heading under both SD and LD conditions (Doi et al. 2004). The *hd3a* and *rft1* double mutant did not undergo heading 200 days after sowing, which indicates the absence of other florigen-like genes in rice genome (Komiya et al. 2003). Delayed heading in rice under LD conditions can be caused by several factors that inhibit *Ehd1*. The *Grain number, plant height and heading date 7* (*Ghd7*) gene, which encodes a CCT (CO, CO-like, and TIMING OF CAB1)-domain protein (Xue et al. 2008), and *DTH8*, which encodes the OsHAP3 subunit of a CCAAT-box binding protein (HAP

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complex) (Dai et al. 2012; Wei et al. 2010; Yan et al. 2011) suppresses the expression of *Ehd1* in LD conditions. *Photoperiod sensitivity-13* (*Se13*) encodes a phytochromobilin synthase involved in phytochrome activity, and the *se13*-deficient mutants were insensitive to day length changes owing to the lack of functional phytochromes (Saito et al. 2011; Yoshitake et al. 2015). Another phytochrome associated gene, *Phytochrome B* (*PHYB*), achieves a similar insensitivity when inactivated by a frame shift (Ishikawa et al. 2011). *Heading date 1* (*Hd1*) is a gene regulator that delays and promotes heading under LD and SD conditions, respectively. Moreover, this regulation is independent of *Ehd1* (Yano et al. 2000). The *Ef7* gene is rice ortholog of *Arabidopsis EARLY FLOWERING 3* (*ELF3*), which delayed heading under both SD and LD conditions. *Ef7* negatively regulates the expression of *Ghd7* (Saito et al. 2012; Yuan et al. 2009). Additionally, *Se14*, which encodes a JumonjiC (JmjC) domain-containing protein, regulated the expression of *RFT1* through *Ehd1* pathway (Yokoo et al. 2014).

Heading time genes have pleiotropic effects on yield components. Different combinations of *Hd1* and *Ehd1* displayed branch number diversity in panicles (Endo-Higashi and Izawa 2011). The deficient mutant of *ghd7* and *dth8* reduces the grain number per panicle (Wei et al. 2010; Xue et al. 2008). There are multiple changes in vegetative organs in transgenic plants bearing defects in mutated *hd3a* (Tamaki et al. 2007). We previously found a reduction in panicles in the *se13* mutant, a decrease in setting rate in the *hd1* mutant, and a decrease in grain number per panicle in *ghd7* mutant (Xu et al. 2014). However, the genetic effects of these genes have not been investigated under an identical genetic background to determine how they affect yield.

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-associated (Cas) systems have been successfully used as an efficient genome editing tool in a numbers of species (Li et al. 2016; Ma et al. 2015; Shan et al. 2013). In the present study, we generated 14 single, double, and triple mutants using CRISPR/Cas9 gene-editing technology and marker-assisted selection (MAS). We accessed the heading time, photoperiod sensitivity, and yield components, revealing distinctly different mutant effects on yield components and heading time. We aimed to gain insight into heading time, with a goal to provide better information and germplasm for rice breeding.

Methods

CRISPR/Cas9 vector construction and plant transformation

The experiment was conducted under the genetic background of Japanese commercial *japonica* cultivar obtained from Kyoto University (Kyoto, Japan). We linked the codon optimized hSpCas9 (Cong et al. 2013) to the maize ubiquitin promoter (UBI) in an intermediate plasmid, and then inserted this expression cassette into a binary pCAMBIA1300 (Cambia, Australia) that contains the hygromycin B phosphotransferase (HPT) gene. Subsequently, we removed the original *BsaI* site in the pCAMBIA1300 backbone using a point mutation kit (Transgen, China). To produce the CRISPR/Cas9 binary vector pBGK032, we inserted a fragment containing an OsU6 promoter (Feng et al. 2013), a negative selection marker gene ccdB flanked by two *BsaI* sites, and a sgRNA derived from pX260 (Cong et al. 2013) into the vector using an in-fusion cloning kit (Takara, Japan). We used Escherichia coli strain DB3.1 to maintain this binary vector (Supplementary Fig. 1). The 23-bp targeting sequences (including PAM) were selected within the target genes, and their targeting specificity was confirmed using a BLAST search against the rice genome (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Hsu et al. 2013). The designed targeting sequences (Fig. 1) were synthesized and annealed to form the oligo adaptors. Vector pBGK032 was digested by *BsaI* and purified using a DNA purification kit (Tiangen, China). A ligation reaction (10 µL) containing 10 ng of the digested pBGK032 vector and 0.05 mM oligo adaptor was conducted and directly transformed into E. coli competent cells to produce CRISPR/Cas9 plasmids. The CRISPR/Cas9 plasmids were introduced into *A. tumefaciens* strain EHA105. Rice transformation was performed as previously described (Nishimura et al. 2006). Genomic DNA was extracted from the transformed plants, and primer pairs flanking the designed target site were used for PCR amplification. We sequenced the 200–500 bp PCR products directly using the Degenerate Sequence Decoding method (Ma et al. 2015).

Analysis of photoperiodic response

The wild type (WT) and mutants were used to evaluate the photoperiod response in the greenhouse. The LD condition was set as 14.5 h light and 9.5 h dark, and the SD condition was set as 9.5 h light and 14.5 h dark. Supplemental artificial light from incandescent lamps (3.24 Wm22 at soil surface) was used for the LD conditions. We transplanted at least five plants for each line, 14 days after sowing. The experiment was conducted on May 21, 2017, with two replicates. We recorded the heading time when the first panicle emerged from the sheath of the flag leaf for each line.

Field experiments

Field experiments were conducted in the experimental farm of Shenyang Agricultural University, Shenyang, China (N41°, E123°) in 2017. The seeds of 72 cultivars were

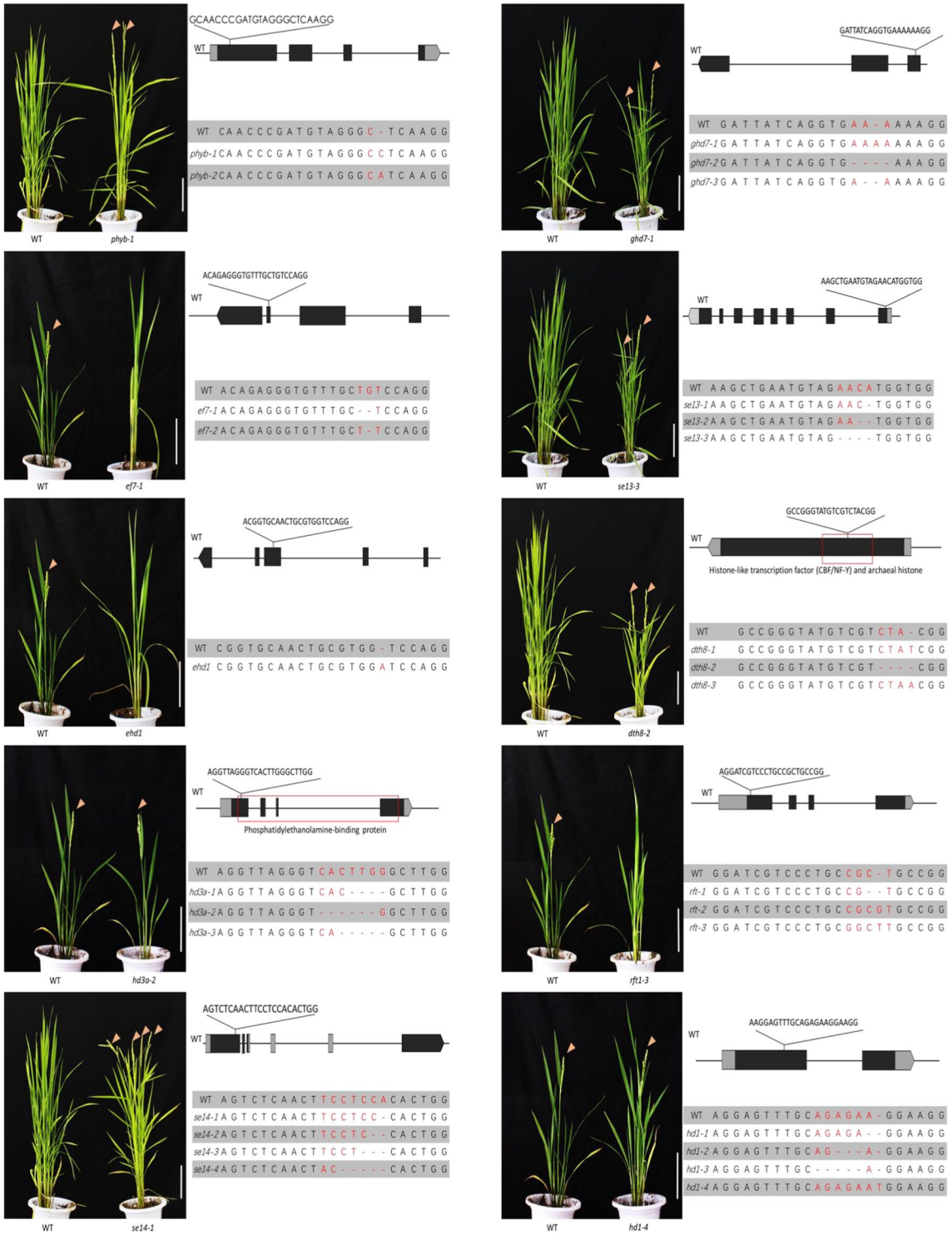

Fig. 1 CRISPR/Cas9-induced single heading time-related mutants and their phenotype. The schematic map of the genomic region of the heading time genes, the sgRNA target sites and the sequence alignment between wild type (WT) and mutants is shown to the right of the plant image. Arrows indicate the emerged panicle. The images were taken when mutants or WT reached heading for each line. The scale bars are equal to 20 cm

obtained from the Academy of Agricultural Sciences for each province. Seeds were sown in the seedling nursery on 24 April, and one seedling was transplanted per hill on 23 May. The experiments were arranged in a randomized block design with three replicates. Each plot was 5.4 m^2 and included 120 plants with planting densities (30 cm × 15 cm

intervals). The cultivation method and field management were as described previously (Li et al. 2018).

DNA extraction, sequencing

We extracted the genomic DNA of 72 cultivars from fresh-frozen leaves using the CTAB method (Doyle 1991). The samples were sequenced on the Illumina HiSeq 2500 according to the manufacturer's instructions, and the sequencing libraries were constructed. We aligned the sequencing reads to the *japonica* reference genome (http://rapdb.dna.affrc.go.jp/) using BWA software (Li and Durbin 2009). In total, 1638.72 Gb of clean data were generated across all 72 cultivars, with approximately 53-fold depth for each cultivar.

Data analysis

The genomic sequences and protein sequences were aligned using ClustalX 2.0.0, and these alignments were used as an input format into TASSEL V2.1. Nucleotide diversity and Tajima's D statistics were calculated using the DnaSP 5.0 program. Statistical analysis was performed using STATISTICA software (StatSoft 1995). The evolutionary relationship among the haplotypes was inferred using the UPGMA method, and phylogenetic analyses were conducted using MEGA5 software.

Results

Heading time gene investigation of receipt for transformation

The *japonica* rice variety Sasanishiki (WT) was used as receipt for transformation. Sasanishiki is a typical Japanese variety which was derived from an ancestor variety 'Gimbozu,' and widely cultivated in Northeast of China before 1980s. Sasanishiki is a major backbone parent for rice breeding in China, and many currently utilized varieties in Northeast China share the genomic pedigree of Sasanishiki (Supplementary Fig. 2). Thus, Sasanishiki is an ideal receipt for transformation not only for function demonstration of heading time genes, but also for the genetic improvement in rice varieties in Northeast China. Ten core heading time genes of Sasanishiki were investigated (Table 1). The results revealed a similar genotype between Sasanishiki and Nipponbare. Only *Hd1* was slightly different, harboring a 43 bp deletion at the first exon of *Hd1* compared to Nipponbare (Supplementary Fig. 3).

Generation of heading time gene mutants using CRISPR/Cas9

To compare the effects of heading time genes on rice reproductive transition and yield components, we mutated ten core heading time genes (Table 1) using CRISPR/Cas9 to specifically induce mutagenesis. We generated the mutant of then heading time genes using CRISPR/Cas9 as described (Li et al. 2017). We examined the mutation efficiency in the T_0 generation, and 20 plants of each mutant were sequenced. Homozygous mutations were determined using Sanger sequence results. Single peaks in Sanger sequence results were considered as homozygous mutation, while the double peaks represented the heterozygous mutations (Supplementary Fig. 4). On average, mutations occurred in 50% of plants, and 13% of sequenced plants had a putative homozygous mutation (Fig. 1 and Table 2). We obtained more than one type of mutation for each heading time gene, except *Ehd1*. For most genes, the different types of mutations exhibited similar phenotypes, except *ghd7-2* and *dth8-2*. Combinations of the *Ghd7*, *Ghd8*/*DTH8,* and *Hd1* genes largely define the ecogeographical adaptation and yield potential of cultivated rice (Zhang et al. 2015), and hence, we crossed the *hd1*, *ghd7,* and *dth8* mutants to generate double and triple mutant lines. We successfully selected *hd1ghd7*, *ghd7dth8*, *hd1dth8* double mutants and

Table 1 Details of the target genes modified in this study

Gene	Molecular function	Mutants	Function	Reference
Se13	Phytochromobilin (PΦB) synthase	Loss photoperiod sensitivity	F	Yoshitake et al. (2015)
PHYB	Similar to phytochrome B	Loss photoperiod sensitivity	F	Ishikawa et al. (2011)
Se14	JMJC protein	Weak photoperiod sensitivity	F	Yokoo et al. (2014)
Hd3a	Florigen	Late flowering and few tiller	F	Komiya et al. (2003)
Ef7	Homolog of the arabidopsis ELF3 protein	Long vegetative growth	F	Saito et al. (2012)
RFT1	Florigen	Late flowering	F	Komiya et al. (2003)
Ehd1	B-type response regulator	Long vegetative growth	F	Doi et al. (2004)
Hd1	Ortholog of Arabidopsis CONSTANS	Weak photoperiod sensitivity	N	Yano et al. (2000)
Ghd7	CCT domain protein	Weak photoperiod sensitivity	F	Xue et al. (2008)
Dth8	Putative HAP3 subunit	Weak photoperiod sensitivity	F	Wei et al. (2010)

F and N represent functional (F) and nonfunctional (N) alleles, respectively

Table 2 Percentage of T_0 plants found with mutations in target gene

Target gene	No. of plants examined	No. of plants with mutations	Mutation rate (%)	Putative homozygous mutations	
				Number	%
Se13	20	12	60.0	3	15.0
PHYB	20	9	45.0	4	20.0
Se14	20	7	35.0	4	20.0
Hd3a	20	8	40.0	2	10.0
Ef7	20	10	50.0	2	10.0
RFT1	20	11	55.0	0	0.0
Ehd1	20	9	45.0	1	5.0
Hd1	20	8	40.0	1	5.0
Ghd7	20	14	70.0	6	30.0
Dth8	20	12	60.0	3	15.0
Total	200	100	50.0	26	13.0

the *hd1ghd7dth8* triple mutant in the F_2 population (Fig. 2). In total, 14 lines (10 single mutants, three double mutants, and one triple mutant) with homozygous mutations were used in this study. Forty plants per mutant were sown in the experimental field of Shenyang Agricultural University (N41°, E123°) on April 23, 2017. The heading time was recorded when the first panicle emerged from the sheath of the flag leaf. Ten early mutants underwent significantly earlier heading than the WT: *phyb*, *se13*, *ghd7*, *dth8*, *se14*, *hd3a, hd1ghd7*, *ghd7dth8*, *hd1dth8,* and *hd1ghd7dth8*. Three late *ef7*, *ehd1,* and *rft1* mutants were significantly delayed in heading time compared with WT.

Characteristics of photoperiod response in mutants

Rice development before heading is divided into two successive phases: The basic vegetative growth phase, which is photoperiod insensitive, and the photoperiod sensitive phase (Yoshitake et al. 2015). The photoperiod sensitive phase is

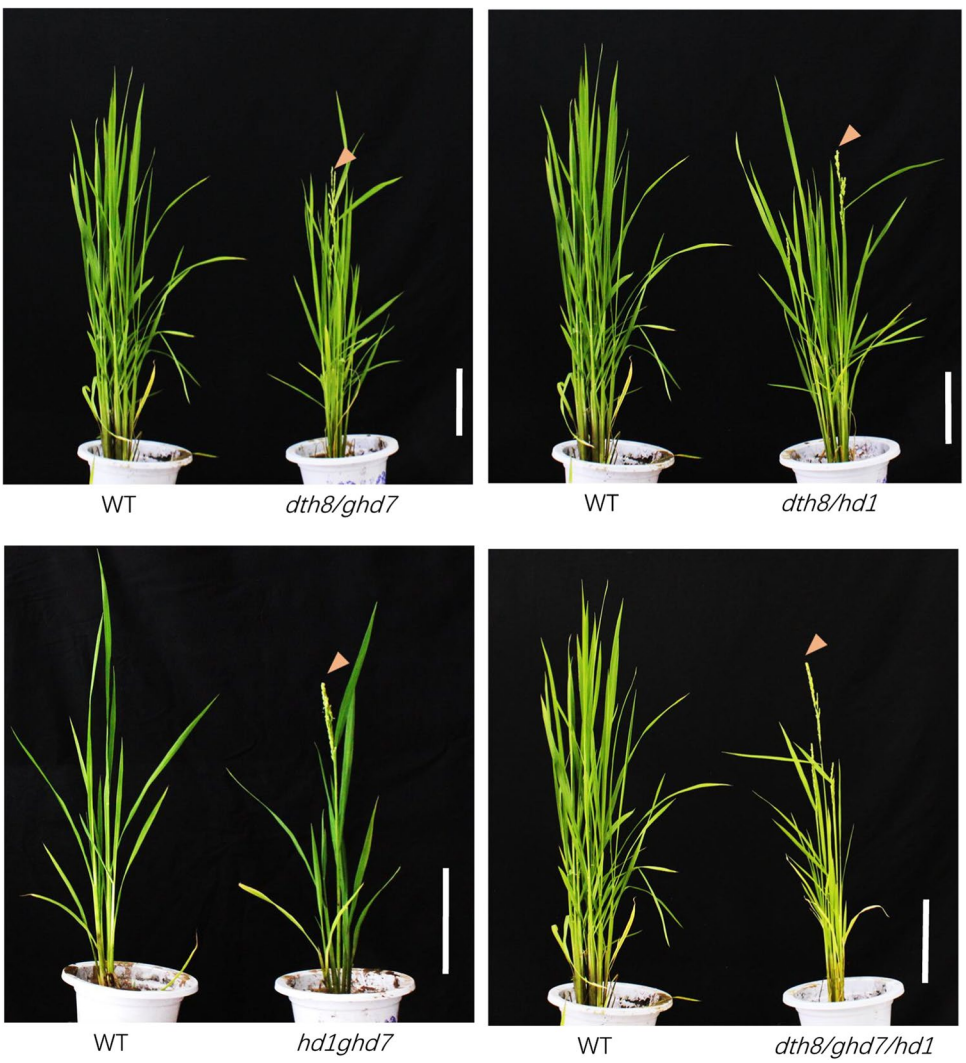

Fig. 2 CRISPR/Cas9-induced double and triple heading time-related mutants and their phenotype. Arrows indicate the emerged panicle. The scale bars are equal to 20 cm

defined as the days to heading (DTH) under LD conditions minus the days to heading under SD conditions. To analyze the photoperiod response of mutants, we planted 14 mutants and the WT under SD and LD conditions. We found that the early mutants had similar basic vegetative growth phase compared to the WT, whereas the photoperiod sensitive phase was significantly shorter than that of the WT. In particular, the *se13* and *phyb* mutants completely lost the photoperiod sensitive phase. The double mutant *ghd7dth8* and triple *hd1ghd7dth8* mutant had an additive effect in the regulation of the photoperiod sensitive phase. On the other hand, late mutants had similar photoperiod sensitive phase compared with the WT, but the basic vegetative growth phase was extended in all but the *rft1* mutant. The *rft1* mutant underwent heading at the same time as the WT under the SD condition, but had a significantly longer photoperiod sensitive phase than that of the WT. Under natural day length, SD and LD conditions, the *hd1* mutant reached heading slightly earlier than the WT (Fig. 3). Interestingly, the *hd3a* mutant showed an earlier heading phenotype under LD and natural day length conditions.

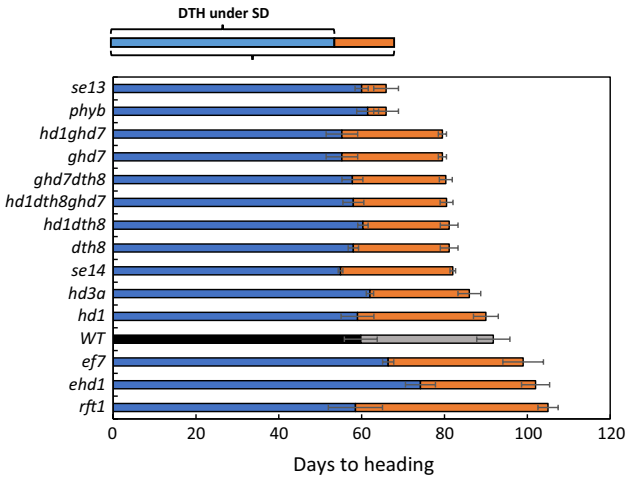

Fig. 3 The heading date of the WT and mutants under SD and LD conditions. The blue color indicates the heading date under SD conditions, the orange color indicates the photoperiod sensitive stage, and the blue color plus the orange color indicates the heading date under LD conditions. The black and gray colors indicate the WT variety

Yield-related traits of mutants

Numerous studies have demonstrated that heading time genes directly regulate yield components. Consequently, we conducted yield-related trait investigations of both mutants and the WT (Table 3). The results showed that the yield increased with an increase in the number of days to heading, but reaching a limit at 115 days to heading (Fig. 4). Early mutants had lower biomass and grain yield per plant; however, the sources of yield loss were different. Among the single mutants, *phyb* exhibited a decrease in the number of grains per panicle and the setting rate, *se13* had fewer panicles, *se14* had a smaller grain size, and *ghd7* and *dth8* showed a decrease in both panicle number and grain number per panicle. The double mutant *dth8/ghd7* and triple mutant *hd1/dth8/ghd7* had severe yield reduction compared with that of *dth8* and *ghd7* single mutants, and even had lower yield than *phyb* mutant and *se13* single mutants. The *hd3a* mutant had reduced yield from decreases in panicle number and setting rate. Among the late mutants, the *ef7* mutant increased grain yield, whereas *rft1* significantly reduced yield by decreasing grain number per panicle. The *ehd1* mutants had a higher grain number per panicle compared with the WT, however, a lower number of panicles placed similar to that of the WT.

Elite allele in mutants

Most of the improved varieties are insensitive to photoperiod by the introduction of the photoperiod-insensitivity gene and can be grown during any season and in most tropical and subtropical countries (Khush 2001). We investigated photoperiod-insensitivity mutants for potential uses in rice breeding. Among the photoperiod insensitive mutants, only *se14* had a yield comparable to the WT, but underwent heading 10 days earlier than the WT under natural field conditions (Fig. 4). An expression analysis showed that *Se14* was ubiquitous but was more highly expressed in the flag leaf, stem, and panicle 5 days after heading and spikelet production, but no significant difference between the WT and *se14* mutant was observed (Supplementary Fig. 5). Our previous study identified *Se14* from artificial γ radiation mutants (Yokoo et al. 2014); however, the natural variation of the *Se14* locus remains unclear. We collected 72 cultivars that were widely released from 1960 to 2000 in the *japonica* cultivated area in China (Supplementary Table 1): 17 cultivars from Heilongjiang Province (HLJ), 19 cultivars from Liaoning Province (LN), 16 cultivars from Shandong Province (SD), and 20 cultivars from Jiangsu Province (JS) (Fig. 5). Then, we sequenced the *Se14* genes for the 72 cultivars, revealing 11 SNPs and 5 INDELs (Fig. 5). Using this information, we constructed 3 haplotypes from the 72 cultivars; all of the cultivars in HLJ belonged to Hap1. There were 17 Hap1, 1 Hap2 and 1 Hap3 among LN cultivars; 9 Hap1 and 7 Hap2 occurred in SD; and 16 Hap1, 3 Hap2, and 1 Hap3 occurred in JS. We compared the heading time and yield of these cultivars in their cultivated area; however, no significant difference was found among haplotypes. As no frame shift occurs among haplotypes compared to the reference genome (Nipponbare and 9311), all three haplotypes likely carry a functional *Se14* allele. In addition to *Se14*, we found that the plants carrying the *dth8* mutant showed significant increases in the 1000-grain weight compared with that of the WT.

Table 3 The yield-related traits of WT and mutants

Lines	PH (cm)	GW (g)	PN	GN	ST	Biomass (g)	Yield (g)
WT	110.16 ± 0.74	22.06 ± 0.01	20.71 ± 0.87	160.13 ± 5.77	0.81 ± 0.01	81.17 ± 2.8	59.77 ± 2.18
phyb	106.67 ± 2.32	22.17 ± 0.05	20.83 ± 1.01	112.33 ± 5.81	0.71 ± 0.05	46.66 ± 3.94	36.77 ± 3.32
ghd7	102.33 ± 1.14	22.51 ± 0.03	17.11 ± 1.51	144.33 ± 3.21	0.77 ± 0.03	67.27 ± 4.82	42.81 ± 4.23
se14	106.17 ± 1.43	20.52 ± 0.02	21.13 ± 1.11	164.67 ± 7.08	0.83 ± 0.02	74.67 ± 4.23	59.26 ± 3.61
hd3a	110.51 ± 1.14	22.51 ± 0.03	17.37 ± 1.51	158.83 ± 3.21	0.77 ± 0.03	80.33 ± 4.82	40.26 ± 4.21
se13	89.17 ± 2.32	22.17 ± 0.05	20.83 ± 1.01	94.51 ± 5.81	0.71 ± 0.05	44.66 ± 3.94	30.86 ± 3.32
rft1	115.33 ± 0.74	22.06 ± 0.02	21.13 ± 0.87	137.25 ± 5.77	0.81 ± 0.01	88.87 ± 2.82	52.01 ± 2.18
hd1	110.16 ± 0.74	22.06 ± 0.03	21.33 ± 0.87	158.31 ± 5.77	0.81 ± 0.01	61.66 ± 2.87	58.08 ± 2.18
ehd1	116.13 ± 0.74	22.91 ± 0.01	19.33 ± 0.87	172.33 ± 5.77	0.81 ± 0.01	83.43 ± 2.13	58.62 ± 2.18
ef7	117.17 ± 0.74	21.01 ± 0.05	23.17 ± 0.87	164.23 ± 5.77	0.74 ± 0.01	90.93 ± 2.33	62.22 ± 2.18
dth8	102.33 ± 3.31	23.61 ± 0.01	17.67 ± 0.55	124.17 ± 12.92	0.82 ± 0.01	62.69 ± 3.91	40.81 ± 3.28
hd1ghd7	101.67 ± 1.14	22.51 ± 0.03	17.13 ± 1.51	144.33 ± 3.21	0.77 ± 0.03	66.79 ± 4.82	42.82 ± 4.21
hd1dth8	102.51 ± 3.31	23.33 ± 0.01	17.51 ± 0.55	138.67 ± 12.92	0.83 ± 0.01	62.87 ± 3.91	47.02 ± 3.28
ghd7dth8	84.67 ± 2.22	23.01 ± 0.02	12.67 ± 1.03	106.16 ± 8.06	0.81 ± 0.02	40.33 ± 4.36	23.89 ± 3.74
hd1dth8ghd7	85.13 ± 2.22	22.91 ± 0.05	12.13 ± 1.03	103.16 ± 7.33	0.81 ± 0.02	39.47 ± 4.36	24.84 ± 3.74

PH plant height, *GW* 1000 grain weight, *PN* panicle number, *GN* grain number per panicle, *ST* setting rate, *Biomass* biomass per plant, *Yield* grain yield per plant

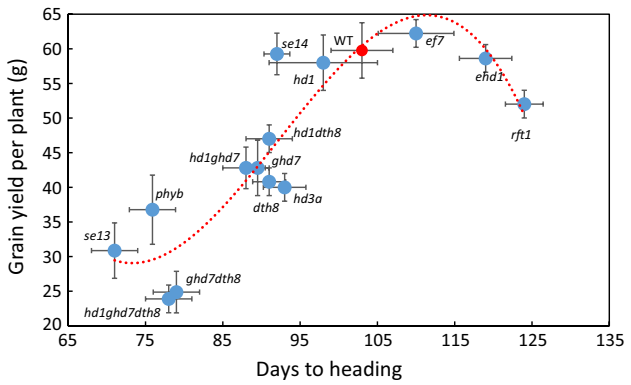

Fig. 4 The relationship between grain yield per plant and heading date and the genotype of each mutant line

However, the decrease in panicle number and grain number per panicle caused the yield reduction.

Discussion

CRISPR/Cas9 is an efficient target mutation method for many species (Cong et al. 2013; Feng et al. 2013; Ma et al. 2015; Shan et al. 2013; Zhou et al. 2018). In this study, we successfully mutated ten heading time genes in a single cultivar. This finding will facilitate the pyramiding of useful genes into a single cultivar for either breeding purposes or the dissection of gene regulatory networks. Novel phenotypes were characterized in this study, which differed from previous works. *Hd1* functions in the promotion of heading under SD conditions and in inhibition under LD conditions (Yano et al. 2000). Sasanishiki carries a mutant *hd1* allele which has a 43 bp deletion at the 3′ terminus of the first exon; our study generated a mutation at the 5′ end of *Hd1*. The *hd1* gene-edited plant reached heading 5 days earlier than Sasanishiki. Thus, the Sasanishiki-type allele of *Hd1* may have a weak function, while the CRISPR/Cas9 type *Hd1* alleles is nonfunctional. *Hd3a* is a florigen of rice and promotes rice heading (Kojima et al. 2002). The present study showed a loss-of-function allele of *hd3a* as an earlier mutant under LD and natural day length conditions, and a later heading under SD conditions. These results indicate that *Hd3a* may mainly promote heading under SD conditions, and the other florigen of rice, *RFT1*, may primarily accelerate heading under LD conditions. However, further research is needed to dissect the molecular mechanisms behind earlier heading in the *hd3a* mutant under LD and natural day length conditions. The phytochrome-related mutants, *se13* and *phyb*, completely lost their photoperiod sensitive phase and exhibited the same heading date under SD and LD conditions. The double mutant *dth8/ghd7* has similar DTH as *se13* and *phyb* mutants, indicating that *ghd7* and *dth8* contribute to the photoperiod sensitivity in Sasanishiki. Our previous study identified earlier heading phenotype *se14* mutant as *Os03g0151300* from mutant pool which was generated by γ radiation treatment. The *se14* mutant had a 23-bp deletion at the first exon of *Os03g0151300*. The transcript experiments showed that *Se14* contains cDNA of two gene locus *Os03g0151300* and *Os03g0151400* (Yokoo et al. 2014). Thus, the *Se14* encodes a protein containing Jumonji N (JmjN), JmjC, and four copies of C2H2-type

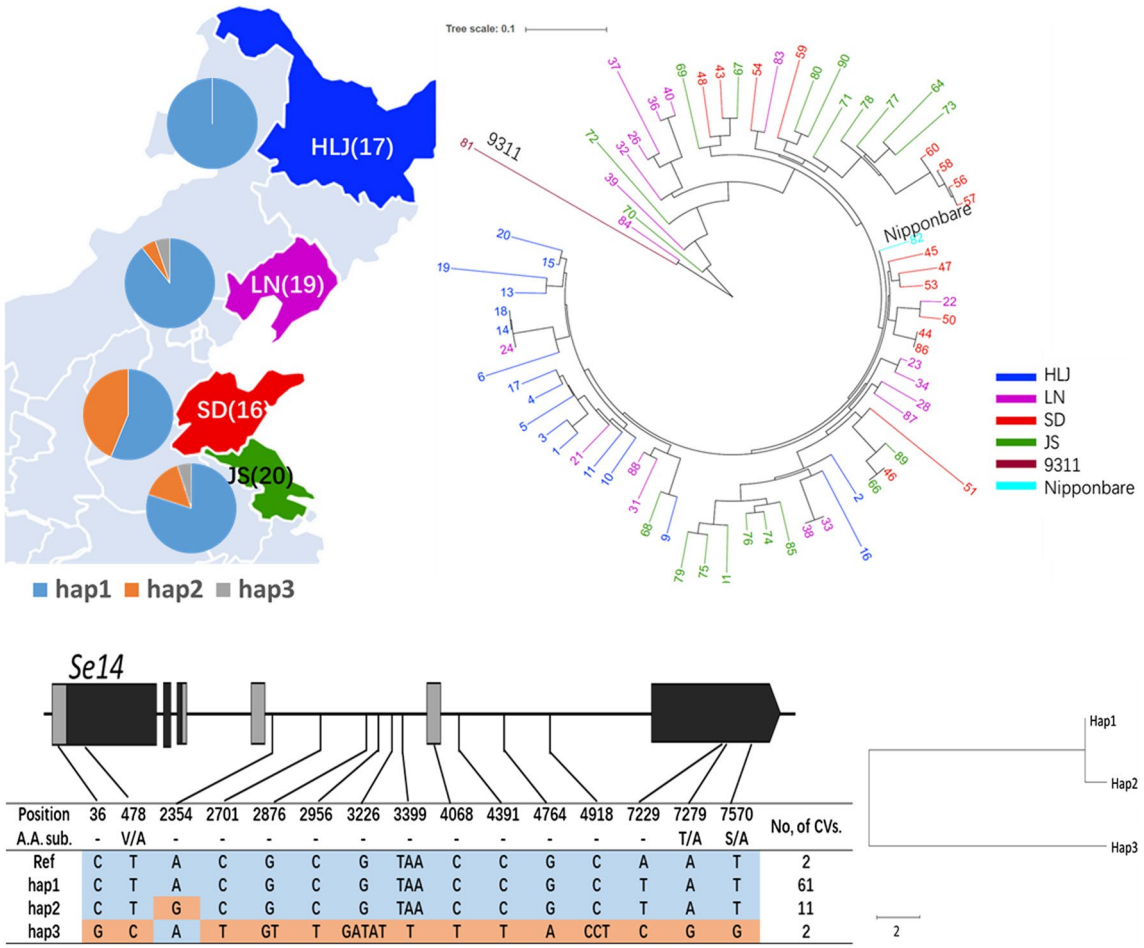

Fig. 5 The variation of the *Se14* locus among 72 cultivars and the haplotype analysis

zinc finger (ZnF) domains, which share high homology with *ELF6* in *Arabidopsis*. The present study confirmed the function of *Se14* in heading time regulation. Moreover, we found that unlike other earlier mutant genes such as *ghd7* and *dth8*, the earlier heading of the *se14* mutant did not cause a decrease in yield.

Late heading is associated with a long growth period, which, in turn, is associated with larger biomass, which may guarantee higher yields. Our study confirms that an insufficient basic vegetative growth phase significantly decreases the yield, and all the early heading mutants had reduced yield (Fig. 4). However, the yield reached a maximum limit with an increase in the number of days to heading. After the yield peaked, the biomass continued to increase with increase in the number of days to heading, whereas the yield began to decrease (Table 2). The temperature of high-latitude areas drops dramatically after August; thus, late heading may cause a plant to suffer from low temperature stress at the pollination or filling stage, leading to a decrease in the setting rate and the 1000 grain weight.

The rice genome contains 20 Jumonji C (jmjC) domain encoding genes, and jmjC domain-containing proteins may function as histone demethylases (Tsukada et al. 2006). The defective *jmj706* mutant caused to increased di- and trimethylations of H3K9, and the floral morphology was changed in mutant (Sun and Zhou 2008). The expression of *JMJ705* is induced by stress signals and during pathogen infection (Li et al. 2013). A previous study demonstrated that *Se14* encodes a jmjC domain-containing protein, and *RFT1* and *Ehd1* were up-regulated in the *se14* mutant, which led to an early heading phenotype (Yokoo et al. 2014). In this study, we found the *se14* mutant underwent heading 10 days earlier than the WT but had the same yield. This elite character will not only save resources in terms of labor, water and arable land but will also improve the adaptive ability of the variety. The sequence analysis of *Se14* among the *japonica* cultivars shows that a nonfunctional *Se14* allele may not yet have been used in rice breeding. However, the function of *se14* needs to be tested under diverse genetic backgrounds to further elucidate its effects. Taken together, these results may provide

new insights into rice breeding for the development of an early heading, high-yielding variety.

Conclusions

We analyzed the relationship between heading time and yield under an identical genetic background by using various heading time mutants developed by a CRISPR/Cas9 system. Heading time is often associated with yield-related traits and biomass, yet we found that yield increased with delayed time to heading, but reaches a maximal level before decreasing. An unexpected elite performance of the *se14* mutant was a striking finding that could be applicable in rice breeding to generate early heading varieties with yield equal to standard varieties.

Author contribution statement QX and ZX conceived the original screening and research plans; YC and MZ performed most of the experiments; QX conceived the project and wrote the article with contributions of all the authors; ZX supervised and complemented the writing. QX agrees to serve as the author responsible for contact and ensures communication. All authors have read and approved the manuscript.

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Availability of data and materials The datasets supporting the conclusions of this article are included within the article and its additional files.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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