Journal of Experimental Botany, Vol. 72, No. 20 pp. 6963–6976, 2021
https://doi.org/10.1093/jxb/erab344 Advance Access Publication 10 July 2021

RESEARCH PAPER

Combinations of *Ghd7*, *Ghd8*, and *Hd1* determine strong heterosis of commercial rice hybrids in diverse ecological regions

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Received 22 March 2021; Editorial decision 19 July 2021; Accepted 20 July 2021

Editor: Joanna Putterill, University of Auckland, New Zealand

Abstract

Heterosis of grain yield is closely associated with heading date in crops. Gene combinations of the major heading date genes *Ghd7*, *Ghd8*, and *Hd1* play important roles in enhancing grain yield and adaptation to ecological regions in rice. However, the predominant three-gene combinations for a specific ecological region remain unclear in both three-line and two-line hybrids. In this study, we sequenced these three genes of 50 cytoplasmic male sterile/maintainer lines, 31 photo-thermo-sensitive genic male sterile lines, and 109 restorer lines. Sequence analysis showed that hybrids carrying strong functional alleles of *Ghd7* and *Hd1* and non-functional *Ghd8* are predominant in three-line hybrids and are recommended for rice production in the subtropics around 30°N/S. Hybrids carrying strong functional *Ghd7* and *Ghd8* and non-functional *Hd1* are predominant in two-line hybrids and are recommended for low latitude areas around 23.5°N/S rich in photothermal resources. Hybrids carrying strong functional *Ghd7* and *Ghd8* and functional *Hd1* were not identified in commercial hybrids in the middle and lower reaches of the Yangtze River, but they have high yield potential in tropical regions because they have the strongest photoperiod sensitivity. Based on these findings, two genic sterile lines, Xiangling 628S and C815S, whose hybrids often head very late, were diagnosed with these three genes, and *Hd1* was targeted to be knocked out in Xiangling 628S and replaced with *hd1* in C815S. The hybrids developed from both modified sterile lines in turn had appropriate heading dates and significantly improved grain yield. This study provides new insights for breeding design to develop hybrids for various regions.

Keywords: Breeding design, gene diagnosis, gene editing, heading date, heterosis, rice (*Oryza sativa* L.), three-gene combination.

Introduction

Heading date (or flowering time) is a crucial trait for the diversification of flowering plants and also a key trait for crop domestication and production. Rice cultivars with different combinations of heading date genes have been planted in a large region from latitude 45°N to latitude 35°S due to different responses of heading date genes to temperature and day length (Monfreda *et al.*, 2008). In general, heading date is correlated with grain yield in cereals (Jung and Müller, 2009; Nelson *et al.*, 2011; Gao *et al.*, 2014; Li *et al.*, 2018; Hu *et al.*, 2019; Zhang *et al.*, 2019*a*; Chen *et al.*, 2020).

Heading date is mainly regulated by photoperiod and temperature in rice (Du *et al.*, 2017). Several major flowering genes with rich natural variations have been cloned and characterized in rice, including *Hd1* (Yano *et al.*, 2000), *Ghd7* (Xue *et al.*, 2008), and *Ghd8/DTH8* (Wei *et al.*, 2010; Yan *et al.*, 2011). All these genes function to delay heading and increase grain yield under natural long-day conditions (LDs), except *Hd1*, the effect of which on controlling heading date depends on interactions with *Ghd7* or *Ghd8* (Du *et al.*, 2017; Zhang *et al.*, 2017, 2019*a*, b).

Haplotype (Hap) or allele analysis showed that there were 10, 11, and 29 Haps of *Ghd7*, *Ghd8*, and *Hd1*, respectively (Zhang *et al.*, 2015). Haps of *Ghd7* and *Ghd8* were classified into functional (F-type) and non-functional (N-type) types; the F-types were further divided into strong functional alleles (S-type) and weak functional alleles (W-type) according to their genetic effects on heading date in near-isogenic lines (NILs). Hap1 and Hap10 of *Ghd7* are S-type with strong effects on heading date, Hap4 is W-type with weak effects, Hap6, which contains a mutation leading to a premature stop codon, and Hap9, which completely loses *Ghd7*, are N-type without effects (Zhang *et al.*, 2015). Similarly, *Ghd8* has two S-types (Hap6 and Hap8), two W-types (Hap2 and Hap4), six N-types (Hap1, Hap3, Hap5, Hap7, Hap9, and Hap11), and one Hap (Hap10) whose function remains unclear (Zhang *et al.*, 2015). Sixteen F-types and 13 N-types were found for *Hd1*, but the F-types were not functionally classified as S- or W-types because NILs were not available at the time (Takahashi *et al.*, 2009; Zhang *et al.*, 2015).

Variations in key flowering genes provide the chance to develop hundreds of varieties with different gene combinations to adapt to their specific ecological regions. Our previous studies showed that different combinations of *Ghd7*, *Ghd8*, and *Hd1* largely defined the eco-geographical adaptation and yield potential of rice cultivars. SSF (a sequential three-gene combination of S-type *Ghd7* and *Ghd8* and F-type *Hd1*) was the combination most sensitive to photoperiod; varieties with the SSF combination headed extremely late and could not produce seeds in normal rice growing seasons in Wuhan (latitude 31°N), China. Cultivars carrying a combination of SSN (a sequential three-gene S-type *Ghd7* and *Ghd8* and N-type *Hd1*) had an appropriate heading date and the highest yield per plant under natural LDs in Wuhan (Zhang *et al.*, 2015). Recently, it was demonstrated that genetic interactions of *Ghd7*, *DTH8/Ghd8*, and *Hd1* controlled the photoperiod heading date under LDs and provided the basis for rice adaptation to a broad range of regions (Zong *et al.*, 2021).

The utilization of heterosis has made great contributions to food security worldwide. The breeding of three-line hybrid rice started in the 1970s when the cytoplasmic male sterile (CMS) line was discovered (Yuan, 1987), and that of two-line hybrid rice started in the 1980s when the photo-thermo-sensitive genic male sterile (P/TGMS) line was identified (Shi, 1981; Shi and Deng, 1986). The three-line hybrid system consists of a male sterile line, a maintainer line, and a restorer line. The CMS line is produced by crossing with the maintainer line, and hybrid rice is produced by crossing a female CMS line with a restorer line. The two-line hybrid system includes a restorer line and a P/TGMS line whose fertility is controlled by day-length and temperature. The hybrid integrates male and female parental genomes to form a new combination of flowering genes, which results in a heading date that varies from both parents. In the development of hybrids, some parents exhibit very good comprehensive phenotypes, but it is very difficult to generate hybrids with reasonable heading dates by crossing with other parents. Considering the wide variation in heading date among the combinations of *Ghd7*, *Ghd8*, and *Hd1* and the integrated genome constitution of hybrids in both three-line and two-line systems, we hypothesized that the three-gene combinations of *Ghd7*, *Ghd8*, and *Hd1* determine strong yield heterosis in commercial three-line and two-line rice hybrids in varied cropping regions in China.

To test this hypothesis, we sequenced the three major flowering genes *Ghd7*, *Ghd8*, and *Hd1* in 50 CMS/maintainer lines, 31 P/TGMS lines, and 109 restorer lines in the present study. We concluded that the hybrid combinations of S_NF_ (including the collective combinations of S^HNF^H, SNF^H, S^HNF, and SNF in hybrids, where the superscripted H means heterozygous at the locus) and S_S_N (including the collective combinations of S^HS^HN, S^HSN, SS^HN, and SSN in hybrids) at the loci of *Ghd7*, *Ghd8*, and *Hd1* in order were predominant for strong heterosis in three-line and two-line hybrids, respectively, in the middle and lower reaches of the Yangtze River of China. Based on these findings, two P/TGMS lines, Xiangling 628S (628S) and C815S, whose hybrids often have very late heading dates, were diagnosed with these three genes. Then, *Hd1* was targeted to be knocked out by gene editing in 628S and replaced with *hd1* by introgression in C815S. Finally, modified 628S (M628S) and C815S (MC815S) were used to successfully generate hybrids with proper heading dates and high grain yields. This research provides new insights into hybrid development by scientifically allocating heading date genes in two parents to pursue a higher grain yield without risk in rice production worldwide.

Materials and methods

Plant materials

A total of 190 parents of rice hybrids were collected. There were 50 CMS/maintainer lines, 31 P/TGMS lines, and 109 restorer lines, including 81 lines commonly used for both two-line and three-line hybrids. 628S is a P/TGMS line bred by Hunan Yahua Seed Industry Research Institute, China (Fu *et al.*, 2010). It can head normally in the middle and lower reaches of the Yangtze River, but its derivate hybrids frequently have very late heading dates, leading to loss of its potential in the breeding of hybrids. Similarly, C815S is a super P/TGMS line bred by Hunan Agricultural University (Tang *et al.*, 2007); it can also head normally, but its hybrid derived by crossing with restorer line 9311 has a very late heading date. The modified 628S (M628S) was generated from 628S by knocking out *Hd1* using a gene editing approach. The modified C815S (MC815S) was developed by introgressing the N-type *hd1* fragment donated by Minghui 63 into the superior P/TGMS line C815S, and genic male sterility was maintained by phenotypic selection of male sterile plants in LDs that would be ratooned under low temperature and short-day conditions for harvesting seeds. The NIL-F_2 population for *Ghd7*, *Ghd8*, and *Hd1* was developed in the Zhenshan 97 background in our previous study (Zhang *et al.*, 2019*a*). NIL ZS97^{Ghd7} (Zhenshan 97 introgressed with S-type *Ghd7*) was developed using Minghui 63 as the donor of S-type *Ghd7*. The hybrids were generated by crossing 628S and M628S with Huazhan, Shuhui 527, and Fuhui 838 and by crossing C815S and MC815S with 9311. Hybrid Shanyou 63 and the modified Shanyou 63 were made by crossing Minghui 63 with Zhenshan 97 or ZS97^{Ghd7}, respectively.

Haplotype analysis of the three major flowering genes

The coding sequences of the three major flowering genes *Ghd7*, *Ghd8*, and *Hd1* in 190 parents were amplified and sequenced as previously reported (Zhang *et al.*, 2015). Sequence contigs were assembled by using the Sequencher 5.4.5 tool. The assembled Haps of these genes were functionally classified as F-type (S-,W-type) or N-type alleles (Takahashi *et al.*, 2009; Zhang *et al.*, 2015).

Generation of Hd1 knockout mutants by CRISPR–Cas9 system

To knock out the target *Hd1*, two sgRNAs (S1 and S2) were first designed based on the CRISPR-P 2.0 tool (Liu *et al.*, 2017), and then the targeting specificity and efficiency were confirmed by BLAST search software on the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and the program RNA Folding Form (http://www.unafold.org/mfold/applications/rna-folding-form-v2.php). The pYL/*Hd1* vector was constructed based on the backbone of pYLCRISPR/Cas9Pubi-H and produced as previously reported (Ma *et al.*, 2015; Zhou *et al.*, 2018). The sgRNAs were driven by the *OsU6b* and *OsU6c* promoters and then ligated to the binary vector pYLCRISPR/Cas9Pubi-H through the Golden Gate cloning method. The codon-optimized *Cas9* in the pYL/*Hd1* plasmid was driven by the *ubiquitin* promoter of maize (*Zea mays* L.). Finally, the pYL/*Hd1* plasmid was introduced into *Agrobacterium tumefaciens* strain EHA105 and transformed into the callus of 628S. The regenerated plants were obtained by effective selection for hygromycin B resistance. The primers used for pYL/*Hd1* construction are listed in Supplementary Table S1.

To screen the *hd1* mutants of 628S, DNA of T_0 transgenic plants was extracted from the leaves and used for transgenic positive identification by using *Cas9*-based primers (Supplementary Table S1). Then, the *Cas9*-positive plants were selected to identify the *hd1* mutants by PCR product sequencing as described in our previous study (Zhou *et al.*, 2018). The primers used for the detection of mutants are listed in Supplementary Table S1.

Field experiments and phenotypic measurement

Twenty-five-day-old seedlings of parents were transplanted to the experimental fields. The transgenic materials were planted separately in the transgenic experimental fields. Twenty plants from each line were planted in two rows with 10 plants per row, with a distance of 16.5 cm between plants in a row and 26.5 cm between rows. The 16 middle plants in each line were used for measurement of traits, including heading date, effective tiller number, seed setting rate, spikelets per panicle, and yield per plant. These measures of agronomic performance were investigated as described previously (Zhang *et al.*, 2015; Zhou *et al.*, 2018). The original Shanyou 63 and modified Shanyou 63 hybrids were sown in the middle of May 2018, the 628S derived and M628S derived hybrids were sown in the middle of May 2019, and the hybrids produced by C815S and MC815S were sown in the middle of May 2020 in the field at Huazhong Agricultural University, Wuhan (31°N latitude, natural LDs), China.

Results

Hundreds of CMS and P/TGMS lines have been released in China (http://www.ricedata.cn/variety/) since the 1970s. Moreover, hundreds of restorer lines have been released to produce hybrids in rice. Eighty-one sterile lines and 109 restorer lines, including the core parental lines that made important contributions to heterosis utilization in different eras, were collected. They were sequenced for *Ghd7*, *Ghd8*, and *Hd1*. The haplotypes for each gene were classified according to their assembled sequences, and are summarized in Supplementary Fig. S1. For ease of description, we designated the combinations with three uppercase letters representing the functional classifications of *Ghd7*, *Ghd8*, and *Hd1*. For instance, SNF means homozygous S-type *Ghd7*, N-type *Ghd8*, and F-type *Hd1* in parents, while S_NF_ means the collective combinations of heterozygous or homozygous S-type *Ghd7*, homozygous N-type *Ghd8*, and heterozygous or homozygous F-type *Hd1* in hybrids.

Three-gene combinations in the CMS female parents

Among the 50 collected CMS/maintainer lines, 26 lines carry S-type *Ghd7* Hap1 or Hap10, while 19 lines carry N-type *Ghd7* Hap9 or Hap11. In addition, five lines carry *Ghd7* Hap2 (Table 1). By comparing the allele sequences with our previous data from 328 varieties (Zhang *et al.*, 2015), we found that a variety with the combination *Ghd7*^{Hap2}SF (*Ghd7* Hap2, S-type *Ghd8* and F-type *Hd1*) exhibited a very late heading at 132.0 d from sowing (sowing on 17 May to heading on 26 September 2012) in Wuhan, indicating that *Ghd7* Hap2 is likely an S-type allele with strong effects similar to those of Hap1 and Hap10 (Supplementary Table S2). *Ghd7* Hap11, a newly found N-type Hap in the present study, loses the whole exon 1 but has the complete exon 2, resulting in a truncated protein. For *Ghd8*, 11 CMS lines including Quan 9311B (A)

Table 1. Three-gene combinations of the CMS (or maintainer) lines included in this study

Line	*Ghd7*	*Ghd8*	*Hd1*	Com.	Type
Qiu B	Hap1	Hap8	Hap1	SSF	WA
Liangfeng B	Hap1	Hap8	Hap5	SSF	WA
Fengtian 1B	Hap1	Hap8	Hap5	SSF	WA
Yufeng B	Hap1	Hap6	Hap2	SSN	WA
Fugui B	Hap1	Hap6	Hap2	SSN	WA
780 B	Hap1	Hap6	Hap2	SSN	WA
Longfeng B	Hap9	Hap6	Hap1	NSF	WA
Mei B	Hap9	Hap8	Hap1	NSF	WA
Bai B	Hap9	Hap8	Hap5	NSF	WA
Hengfeng B	Hap1	Hap1	Hap1	SNF	WA
Huaxiang B	Hap1	Hap5	Hap5	SNF	D
Hua 1517B	Hap1	Hap12	Hap1	SNF	WA
Zhongzhe B	Hap1	Hap5	Hap1	SNF	WA
Huafeng B	Hap2	Hap12	Hap1	SNF	WA
Ruofeng B	Hap10	Hap12	Hap5	SNF	WA
Wufeng A	Hap1	Hap11	Hap5	SNF	WA
Tianfeng A	Hap1	Hap11	Hap5	SNF	WA
Hua 176 A	Hap1	Hap11	Hap5	SNF	WA
Taifeng B	Hap1	Hap12	Hap5	SNF	IP
Guang 8B	Hap1	Hap5	Hap2	SNN	WA
Yexiang A	Hap1	Hap12	Hap2	SNN	IP
Yixiang 1B	Hap1	Hap11	Hap2	SNN	D
Xiangai B	Hap1	Hap5	Hap10	SNN	WA
Luohong 5A	Hap1	Hap5	Hap2	SNN	HL
Jiahong 2A	Hap1	Hap11	Hap2	SNN	HL
E578 B	Hap1	Hap5	Hap2	SNN	WA
E283 B	Hap1	Hap5	Hap2	SNN	WA
Bo IIIB	Hap1	Hap11	Hap2	SNN	WA
Zhongzhe 2B	Hap1	Hap5	Hap2	SNN	WA
Huagui B	Hap1	Hap12	Hap2	SNN	WA
Hongnuo 1A	Hap2	Hap5	Hap2	SNN	HL
Yuetai A	Hap2	Hap5	Hap2	SNN	HL
Gang 46A	Hap2	Hap11	Hap2	SNN	G
Luohong 103A	Hap2	Hap11	Hap2	SNN	HL
Jufeng 2A	Hap11	Hap8	Hap2	NSN	WA
Quan 9311B	Hap11	Hap8	Hap2	NSN	IP
Zhenshan 97A	Hap9	Hap11	Hap1	NNF	WA
V20 A	Hap9	Hap11	Hap1	NNF	WA
Bo A	Hap9	Hap11	Hap1	NNF	WA
Longtefu A	Hap9	Hap11	Hap1	NNF	WA
Jin 23A	Hap9	Hap11	Hap1	NNF	WA
Chaoyang 1B	Hap9	Hap5	Hap1	NNF	WA
II-32A	Hap9	Hap11	Hap1	NNF	IP
You IA	Hap9	Hap11	Hap1	NNF	IP
Zhong 9A	Hap9	Hap11	Hap1	NNF	IP
Xieqingzao A	Hap9	Hap11	Hap5	NNF	DA
Tianxiang B	Hap9	Hap12	Hap2	NNN	WA
Luohong 3A	Hap9	Hap5	Hap2	NNN	HL
Luohong 4A	Hap9	Hap5	Hap2	NNN	HL
Luohong 7A	Hap9	Hap5	Hap2	NNN	HL

Com., three-gene combination. S-type (S) and N-type (N) Haps of *Ghd7* and *Ghd8* are shown in purple and red, respectively. F-type (F) and N-type Haps of *Hd1* are shown in black and red, respectively. Haplotype classification was performed according to our previous report (Zhang *et al.*, 2015). The three SSF, three SSN and three NSF CMS lines shown in bold were bred in Guangxi, China (23°N latitude, natural short day conditions). D, Dissi d52/57; DA, Dwarf Abortion; G, Gambiaca; HL, Honglian; IP, Indonesia Paddy; WA, Wild Abortion.

and Jufeng 2A and the nine lines from Guangxi carry S-type Hap6 or Hap8, while the remaining 39 lines carry Hap1, Hap5, Hap11, or Hap12 (Table 1), which were previously reported as N-type *Ghd8* (Yan *et al.*, 2011; Zhang *et al.*, 2015). Twenty-six lines carry F-type *Hd1* Hap1 or Hap5, while 24 lines carry N-type *Hd1* Hap2 or Hap10 (Table 1).

Overall, for three-gene combinations, 10, 15, 10, 3, 3, 3, 4, and 2 CMS lines carry NNF, SNN, SNF, NSF, SSF, SSN, NNN, and NSN, respectively (Supplementary Table S3). Moreover, the eight CMS lines most widely used in the 1980s–2000s, Zhenshan 97A, V20A, Bo A, II-32A, Jin 23A, Xieqingzao A, Longtufu A, and Zhong 9A, carry the same combination NNF, while the eight HL-CMS lines carry combinations SNN or NNN. The newly developed CMS lines Quan 9311A(B) and Jufeng 2A carry the NSN combination (Table 1).

Three-gene combinations in the P/TGMS female parents

The 31 collected P/TGMS lines included extensively used PGMS Nongkeng 58S derivatives (such as Peiai 64S, Chun 6S, Guangzhan 63S, and Guangzhan 63-4S), TGMS Zhu 1S and its derivatives (such as Xiangling 628S and Jin 4128S), and P/TGMS lines such as C815S, Y58S, and Longke 638S. Sixteen lines carry S-type *Ghd7* Hap1, Hap2, or Hap10, four lines carry W-type Hap4, and 11 lines carry N-type Hap11 (Table 2). Two lines carry S-type *Ghd8* Hap6, six lines carry W-type Hap2, and 23 lines carry N-type Hap1 or Hap5 (Table 2). Eight lines carry F-type *Hd1* Hap1, Hap5, or Hap7, while 23 lines share the same N-type Hap2 (Table 2).

Taken together, the results show that 16 lines have the combination SNN, six lines are NNF, and the remaining nine lines have other three-gene combinations (NSF, NSN, WWN, WNN, NWN and WWF) at low frequencies (Supplementary Table S3).

Three-gene combinations in the restorer male parents

The 109 collected restorer lines included 81 restorer lines used for both three-line and two-line hybrid systems and 28 restorer lines exclusively used for only the two-line system. The lines widely used for generating both three-line and two-line hybrids were included, such as Minghui 63, Shuhui 527, Fuhui 838, Chenghui 727, Minghui 86, Gui 99, R402, Xianhui 207, Guanghui 998, Huazhan, Wushansimiao, Yuenongsimiao, 9311, and so on.

Among the 81 restorer lines for both hybrid systems, 76 lines carry S-type *Ghd7*, two lines carry W-type *Ghd7*, two lines carry N-type *Ghd7*, and one line carries F-type Hap3 for which it is unclear whether it acts as S-type or W-type (Table 3). Except for four S-type lines and five W-type lines of *Ghd8*, the remaining 72 lines carry N-type *Ghd8* (Table 3). All except five lines carry N-type *Hd1* (Table 3). Taken together, the results show that 64 lines belong to SNN, four lines carry SSN, five lines carry SWN, three lines carry SNF, and the remaining five lines belong to WNF, WNN, NNF, NNN, or FNN (Supplementary Table S3). Among the 28 restorer

Table 2. Three-gene combinations of the P/TGMS lines included in this study

Line	*Ghd7*	*Ghd8*	*Hd1*	Com.	Line	*Ghd7*	*Ghd8*	*Hd1*	Com.
Peiai 64S	Hap10	Hap5	Hap2	SNN	Y58S	Hap4	Hap5	Hap2	WNN
Xinan S	Hap2	Hap5	Hap2	SNN	Longke 638S	Hap11	Hap6	Hap2	NSN
Nuo 56S	Hap1	Hap5	Hap2	SNN	7188S	Hap4	Hap2	Hap2	WWN
Yi S	Hap1	Hap5	Hap2	SNN	Hunong 5S	Hap4	Hap2	Hap2	WWN
Huhan 88S	Hap1	Hap1	Hap2	SNN	Shen 08S	Hap11	Hap2	Hap2	NWN
Hua 6040S	Hap1	Hap5	Hap2	SNN	Chun 6S	Hap11	Hap2	Hap2	NWN
Hua 634S	Hap1	Hap5	Hap2	SNN	Chun 199S	Hap11	Hap2	Hap2	NWN
33S	Hap1	Hap5	Hap2	SNN	Xiangling 628S	Hap11	Hap6	Hap5	NSF
Guangzhan 63-4S	Hap2	Hap5	Hap2	SNN	Nongkeng 58S	Hap4	Hap2	Hap7	WWF
Guangzhan 63S	Hap2	Hap5	Hap2	SNN	HD9802S	Hap11	Hap5	Hap5	NNF
Hua 86S	Hap2	Hap5	Hap2	SNN	C815S	Hap11	Hap5	Hap5	NNF
Hua 8S	Hap2	Hap5	Hap2	SNN	Jin 4128S	Hap11	Hap5	Hap5	NNF
Huhan 74S	Hap2	Hap1	Hap2	SNN	Zhu 1S	Hap11	Hap5	Hap5	NNF
6303S	Hap2	Hap5	Hap2	SNN	Hua 5113S	Hap11	Hap5	Hap1	NNF
Hua 6421S	Hap2	Hap5	Hap2	SNN	Hua 448S	Hap11	Hap5	Hap1	NNF
Hua 1228S	Hap2	Hap5	Hap2	SNN					

Com., three-gene combination. S-type (S), W-type (W) and N-type (N) Haps of *Ghd7* and *Ghd8* are shown in purple, green, and red, respectively. F-type (F) and N-type Haps of *Hd1* are shown in black and red, respectively.

lines used in only the two-line system, all lines carry S-type *Ghd7*, and N-type *Hd1*; seven lines carry S-type *Ghd8* Hap8, two lines carry W-type Hap2, while the remaining 19 lines carry N-type *Ghd8* (Table 3). Thus, there are 19 SNN lines, seven SSN lines, and two SWN lines. In summary, among the 109 investigated restorer lines, 83 lines belong to SNN, 11 lines carry SSN, seven lines carry SWN, three lines carry SNF, and the others carry WNF, WNN, NNF, FNN, or NNN (Supplementary Table S3).

Three-gene combinations in the hybrids

For the three-line system, NNF is the predominant genotype in the WA-, DA-, and IP-CMS lines, and SNN is present in high frequency in the restorer lines represented by Minghui 63 and Huazhan (Tables 1, 3). Thus, the combination S^HNF^H (the superscripted uppercase H means heterozygous allele) is predominant in three-line hybrids, especially in those produced by WA-CMS parents. For instance, the most widely cultivated hybrid, Shanyou 63, is a combination obtained by crossing the WA-CMS line Zhenshan 97A (NNF) with Minghui 63 (SNN). Moreover, SNN and NNN are carried by the eight investigated HL-CMS lines (Table 1), while SSN is shared by restorer lines such as 9311 and Chenghui 9348 (Table 3). Thus, S^HS^HN and SS^HN are the main combinations in hybrids produced by the HL-CMS system. For example, HongLianyou 6 is produced by crossing the HL-CMS line Yuetai A (SNN) with 9311 (SSN).

For the two-line system, SNN and NNF are present in high frequency in the P/TGMS lines, represented by Peiai 64S and C815S, respectively (Table 2). SSN and SNN are predominant in the corresponding restorer lines, represented by 9311 and Huazhan, respectively (Table 4). Therefore, SS^HN (SNN crosses with SSN) and S^HNF^H (SNN crosses with NNF) are the main combinations in two-line hybrids. For instance, the super two-line hybrid YangLiangyou 6, between Guangzhan 63-4S (SNN) and 9311 (SSN), carries the combination SS^HN. The super two-line hybrid CLiangyouHuazhan, between C815S (NNF) and Huazhan (SNN), carries the S^HNF^H combination.

No combinations of S_S_F_ in the commercial hybrids for the middle and lower reaches of the Yangtze River

Eight S_S_F_ combinations (SSF, S^HSF, SS^HF, SSF^H, S^HS^HF, S^HSF^H, SS^HF^H, $S^HS^HF^H$) are expected to be generated in either a three-line or two-line system if female parents randomly cross with male parents (Supplementary Table S3). Regardless of the restriction of the restoration–maintenance relationships in the three-line system, theoretically, 4050 crosses could be generated among the 50 CMS and 81 restorer parents (Supplementary Table S3). Among the 4050 crosses, 563 crosses (13.9%) would gain the S_S_F_ combinations (Supplementary Table S4). Accordingly, 184 crosses (5.4%) with the S_S_F_ combinations could be produced among the possible 3379 crosses by 31 P/TGMS and 109 restorer parents (Supplementary Tables S3, S4). However, there are no commercial hybrids with S_S_F_ combinations, except for those produced by the nine SSF, SSN, or NSF CMS lines in Guangxi, China (23°N latitude, natural short day conditions) (Table 1; Supplementary Table S5). It is inferred that the hybrids with S_S_F_ combinations head very late and pose high risks in rice production. Therefore, they were eliminated in the hybrid kingdom in China except the lower latitude regions like Guangxi and Hainan Island. To confirm this inference, we randomly selected eight plants without heading on 1 October from various rice fields in Wuhan, Central China, which were likely hybrids generated by pollination contamination. These plants are called 'Lao-lai-qing' locally, which means that they are sufficiently old but do not flower (Supplementary Fig. S2). Gene diagnosis revealed that three, three, one, and one plants

Table 3. Three-gene combinations of the restorer lines included in this study

Line	*Ghd7*	*Ghd8*	*Hd1*	Com.	Line	*Ghd7*	*Ghd8*	*Hd1*	Com.
81 restorer lines used for both three- and two-line hybrid systems									
9311	Hap1	Hap8	Hap2	SSN	Chenghui 727	Hap1	Hap5	Hap2	SNN
Chenghui 9348	Hap1	Hap8	Hap2	SSN	Luhui 17	Hap1	Hap9	Hap2	SNN
Gui 33	Hap2	Hap6	Hap2	SSN	Yunhui 72	Hap1	Hap11	Hap2	SNN
Gui 169	Hap1	Hap6	Hap10	SSN	Yahui 2115	Hap1	Hap5	Hap2	SNN
Milyang 23	Hap1	Hap2	Hap2	SWN	Yihui 4245	Hap1	Hap12	Hap2	SNN
Ninghui 21	Hap1	Hap2	Hap4	SWN	Yuzhenxiang	Hap1	Hap12	Hap2	SNN
C418	Hap1	Hap2	Hap3	SWN	Huanghuazhan	Hap2	Hap1	Hap2	SNN
R273	Hap1	Hap2	Hap2	SWN	R1988	Hap2	Hap1	Hap3	SNN
R647	Hap1	Hap2	Hap2	SWN	Yuexiangzhan	Hap2	Hap11	Hap2	SNN
Hanhui 3	Hap1	Hap5	Hap1	SNF	Ce 64-7	Hap1	Hap5	Hap2	SNN
Gui 362	Hap1	Hap5	Hap1	SNF	CDR22	Hap1	Hap5	Hap2	SNN
R128	Hap2	Hap5	Hap5	SNF	R644	Hap1	Hap5	Hap2	SNN
Teqing	Hap10	Hap5	Hap2	SNN	Zhong413	Hap2	Hap11	Hap2	SNN
Shuhui 527	Hap1	Hap5	Hap2	SNN	Mianhui 725	Hap1	Hap5	Hap2	SNN
Minghui 63	Hap1	Hap5	Hap2	SNN	R6326	Hap1	Hap5	Hap2	SNN
Fuhui 838	Hap1	Hap5	Hap2	SNN	Guanghui 998	Hap1	Hap5	Hap2	SNN
Huazhan	Hap2	Hap1	Hap3	SNN	R5113	Hap1	Hap5	Hap2	SNN
Yuenongsimiao	Hap2	Hap1	Hap3	SNN	Gui 339	Hap1	Hap5	Hap2	SNN
Wushansimiao	Hap2	Hap1	Hap3	SNN	Fuhui 305	Hap1	Hap5	Hap2	SNN
Milyang 46	Hap1	Hap5	Hap2	SNN	Ganghui 38	Hap1	Hap5	Hap2	SNN
Minghui 70	Hap1	Hap5	Hap2	SNN	E331	Hap2	Hap1	Hap3	SNN
Luhui 615	Hap1	Hap5	Hap2	SNN	Gui 649	Hap2	Hap5	Hap2	SNN
Xianhui 207	Hap1	Hap5	Hap2	SNN	R96	Hap2	Hap5	Hap2	SNN
IR 24	Hap1	Hap5	Hap2	SNN	R404	Hap1	Hap12	Hap2	SNN
IR 26	Hap1	Hap5	Hap2	SNN	R191	Hap2	Hap1	Hap3	SNN
IR 36	Hap1	Hap5	Hap2	SNN	Gui 168	Hap1	Hap1	Hap2	SNN
IR 64	Hap1	Hap5	Hap2	SNN	Minghui 164	Hap1	Hap1	Hap2	SNN
Ganghui 12	Hap1	Hap5	Hap2	SNN	Fuhui 639	Hap1	Hap5	Hap2	SNN
Ruihui 6	Hap1	Hap5	Hap2	SNN	R2015	Hap1	Hap5	Hap2	SNN
Fuhui 676	Hap1	Hap5	Hap2	SNN	Gui 539	Hap1	Hap1	Hap2	SNN
R323	Hap1	Hap5	Hap2	SNN	Guihui 717	Hap2	Hap5	Hap18	SNN
R6	Hap1	Hap5	Hap2	SNN	Guihui 089	Hap1	Hap5	Hap2	SNN
Gui 99	Hap1	Hap5	Hap2	SNN	Gui 826	Hap2	Hap5	Hap2	SNN
Wan 3	Hap1	Hap5	Hap2	SNN	Ce 253	Hap2	Hap5	Hap2	SNN
Gui 1025	Hap1	Hap5	Hap2	SNN	R6547	Hap1	Hap5	Hap2	SNN
Guihui 553	Hap1	Hap5	Hap2	SNN	Hang 1	Hap4	Hap5	Hap1	WNF
Guihui 1561	Hap1	Hap5	Hap2	SNN	Minghui 86	Hap4	Hap5	Hap2	WNN
Guihui 110	Hap1	Hap5	Hap2	SNN	Chenghui 448	Hap3	Hap5	Hap2	FNN
Ce 258	Hap2	Hap5	Hap2	SNN	Gui 630	Hap9	Hap5	Hap5	NNF
R207	Hap1	Hap5	Hap2	SNN	R822	Hap11	Hap1	Hap3	NNN
R402	Hap1	Hap5	Hap2	SNN					
28 restorer lines only used in two-line hybrid system									
Xinhui 1998	Hap1	Hap8	Hap4	SSN	Changhui 70	Hap2	Hap1	Hap2	SNN
Lixiang 85	Hap1	Hap8	Hap2	SSN	Nongxiang 42	Hap1	Hap5	Hap3	SNN
Anxuan 6	Hap1	Hap8	Hap2	SSN	HN38	Hap1	Hap5	Hap2	SNN
Hua 743	Hap1	Hap8	Hap2	SSN	R2303	Hap1	Hap5	Hap2	SNN
Hua 655	Hap1	Hap8	Hap2	SSN	Runzhuyinzhan	Hap2	Hap1	Hap3	SNN
JM20267	Hap1	Hap8	Hap3	SSN	Wanxiangsimiao	Hap2	Hap11	Hap2	SNN
Hua 3234	Hap1	Hap8	Hap2	SSN	Jiafuzhan	Hap2	Hap5	Hap2	SNN
JM2071	Hap1	Hap2	Hap2	SWN	Nongxiang 16	Hap2	Hap5	Hap2	SNN
JM2072	Hap1	Hap2	Hap2	SWN	R308	Hap2	Hap1	Hap2	SNN
Huazhen 319	Hap2	Hap1	Hap2	SNN	Xiangyaxiangzhan	Hap2	Hap5	Hap2	SNN
Huazhen 371	Hap2	Hap1	Hap3	SNN	Meixiangzhan 2	Hap2	Hap5	Hap2	SNN
Huazhen 557	Hap2	Hap1	Hap2	SNN	Meixiangxinzhan	Hap2	Hap12	Hap3	SNN
IR72	Hap1	Hap5	Hap2	SNN	Ce 758	Hap1	Hap5	Hap2	SNN
IR50	Hap1	Hap5	Hap2	SNN	Nongxiang 39	Hap1	Hap12	Hap3	SNN

Com., three-gene combination. S-type (S), W-type (W) and N-type (N) Haps of *Ghd7* and *Ghd8* are shown in purple, green, and red, respectively. F-type (F) and N-type Haps of *Hd1* are shown in black and red, respectively. *Ghd7* Hap3 is an F-type allele which could not be classified as S- or W-type here.

Table 4. The agronomic performance of various genotypes under natural LDs in Wuhan, 2019

Genotype	Comb.	HD (d)	PTN	SPP	SR (%)	YD (g)
628S	NSF	70.6±1.0	9.2±1.8	141±18.4	—	—
M628S	NSN	77.0±1.4**	11.4±2.2	117±16.1	—	—
628S/FH838	$S^HS^HF^H$	120.7±1.0	11.5±2.3	176.7±21.7	79.7±4.3	41.0±8.3
M628S/FH838	S^HS^HN	82.4±3.0**	14.6±3.7*	230.2±20.2**	88.7±2.8**	63.2±3.5**
FH838	SNN	87.2±2.5	9.4±2.1	185.5±12.7	84.0±4.3	39.9±9.8
628S/HZ	$S^HS^HF^H$	123.4±1.1	11.0±3.4	214.3±13.9	63.5±2.5	33.6±11.1
M628S/HZ	S^HS^HN	85.9±0.8**	12.1±1.3	214.2±4.9	84.8±1.5**	57.9±8.4**
HZ	SNN	83.5±1.9	10.1±1.5	246.6±26.4	78.7±4.2	30.5±5.8
628S/SH527	$S^HS^HF^H$	121.6±0.9	8.9±2.3	170.9±28.3	78.9±3.6	32.9±9.7
M628S/SH527	S^HS^HN	80.6±2.4**	12.4±2.4**	177.1±22.4	88.0±2.3**	48.1±6.3**
SH527	SNN	86.8±1.6	9.5±1.2	192.0±15.5	85.0±2.2	43.2±4.8
Fengliangyou 4	SS^HN	87.3±1.3	8.7±1.5	216.2±24.7	87.5±7.5	44.3±5.4

Com., three-gene combination. HD, heading date; PTN, productive tiller number; SPP, spikelet number per panicle; SR, seed setting rate; YD, yield per plant. —, without measurement of the traits. Asterisks represent significant difference between the 628S and M628S, or their hybrids; *P≤0.05, **P≤0.01. Values are mean ±SD (n=16).

carry combinations SSF^H, SS^HF, SS^HF^H, and $S^HS^HF^H$, respectively (Supplementary Table S6). These results indicate that the S_S_F_ combinations lead to extremely delayed heading or even no heading, which might be the reason for the absence of S_S_F_ combinations in commercial hybrids for the middle and lower reaches of the Yangtze River.

Gene diagnosis of 628S and C815S

It was reported that the hybrids produced by crossing 628S with Fuhui 838 and Shuhui 527 showed extremely late flowering in the middle and lower reaches of the Yangtze River under natural LDs (Fu *et al.*, 2010). In our breeding process, the hybrid between C815S and 9311 also exhibited extremely delayed heading under natural LDs. To determine why the heading dates of hybrids developed with 628S and C815S as the female parents were so delayed, we analysed the three-gene combination of 628S and C815S. The gene diagnosis results showed that 628S carries N-type *Ghd7*, S-type *Ghd8*, and F-type *Hd1*, while C815S harbors N-type *Ghd7* and *Ghd8* and F-type *Hd1*. Thus, 628S and C815S carry the NSF and NNF combinations, respectively (Supplementary Table S7). Since the restorer lines for two-line hybrids frequently harbor S-type *Ghd7* (Supplementary Table S3), such as Fuhui 838 and Shuhui 527, while 9311 carries both S-type *Ghd7* and *Ghd8* (Supplementary Table S7), the 628S derived hybrids often carry the $S^HS^HF^H$ combination, and the hybrid between C815S and 9311 also possesses $S^HS^HF^H$. This combination has a very late heading date. To promote heading of 628S and C815S derived hybrids, we destroyed F-type *Hd1* in 628S and C815S.

Phenotype of Hd1 *knockout mutants of 628S*

We generated 100 *Hd1*-edited T_0 transgenic plants using the CRISPR–Cas9 system, among which 26 individuals were positive transgenic plants detected by PCR with *Cas9*-based primers. Eight of the 26 positive transgenic individuals were further analysed by sequencing *Hd1* PCR-products, and seven mutants were identified with InDels or SNPs. Of these, two homozygous mutants, #9 and #99, were used for further studies (Fig. 1A). Mutant #9 has 7-bp and 40-bp deletions in the two target sites S1 and S2, respectively. Mutant #99 has an A insertion and a G deletion in S1 and S2, respectively (Fig. 1A). The mutations in both mutants #9 and #99 cause frame shift of the CCT domain in *Hd1* and lead to loss of function. The T_1 transgenic plants M628S with mutated *hd1* (NSN) headed approximately 7.0 d later than the 628S plants (NSF) under natural LDs (Table 4; Fig. 1B, C). This delayed heading of M628S (NSN) was not in accordance with the report that *DTH8/Ghd8* interacted with *Hd1* to delay the rice heading date (Du *et al.*, 2017), while in our previous study, NIL-F_4 lines with NSN combination had a heading date of 75.7 d, about 4.0 d later than NSF lines (71.9 d) in N-type *Ghd7.1* background (Zhang *et al.*, 2019*a*). Thus, we sequenced *Ghd7.1* from 628S. As expected, 628S carries N-type *Ghd7.1* Hap2 that was previously reported to have an 8-bp deletion causing frameshift mutation (Yan *et al.*, 2013).

Improved performances of hybrids derived from the M628S

Both 628S and M628S were crossed with three widely used restorer lines (Fuhui 838, Shuhui 527, and Huazhan) to generate hybrids. As expected, all the hybrids from M628S exhibited suitable heading dates (80.6–85.9 d) in Wuhan, under natural LDs, heading approximately 40.0 d earlier than their counterparts from 628S (120.7–123.4 d), because the M628S derived hybrids have the S^HS^HN combination rather than $S^HS^HF^H$ (Table 4; Fig. 2). Significant increases in seed setting rate and yield were detected in all modified hybrids (Table 4; Fig. 2). For example, the grain yield per plant of the hybrid between Fuhui

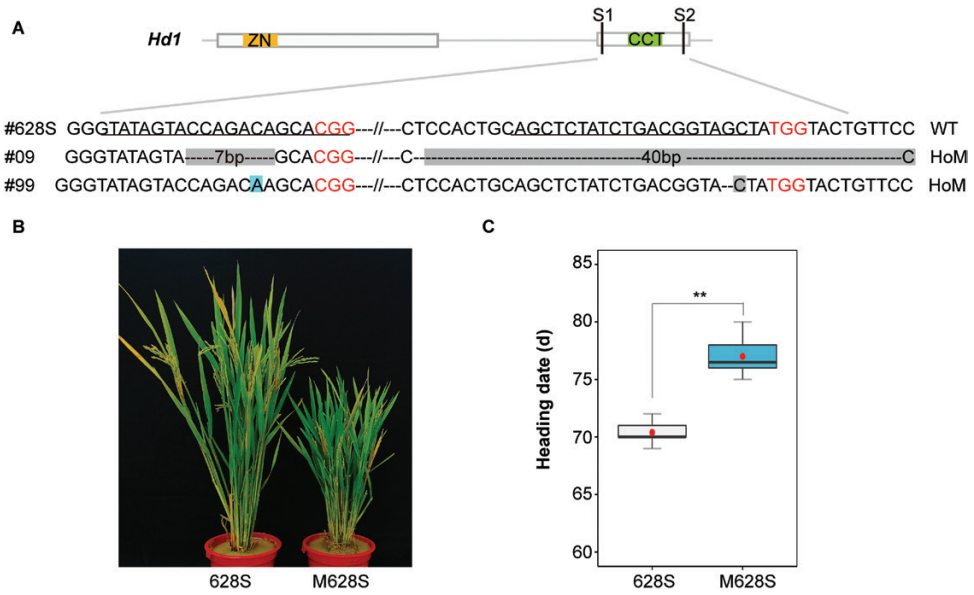

Figure 1. Heading date performance of homozygous *hd1* mutants under LDs. (A) Sequences showing the mutations in two homozygous *hd1* mutants (#09 and #99). The regions encoding Zn (zinc finger) and CCT (CO, CO-LIKE, and TIMING OF CAB1) domains are indicated in *Hd1*. S1 and S2 are the two sgRNA sites. The short sequences underlined are the sgRNA targets, while the bases in red are the Protospacer-adjacent motifs. Insertion and deletion mutations are filled in sky blue and gray, respectively. HoM, homozygous mutants; WT, wild-type plants. (B) Transgenic plants of mutated *hd1* (M628S) and 628S. (C) Heading dates of M628S and 628S in the T_1 generation. **Significant difference, $P \leq 0.01$ (n=16).

838 and M628S was 63.2 g, which was much higher than the 41.0 g observed for the hybrid between Fuhui 838 and 628S. The yield increase was comprehensively contributed by the increased seed setting rate (from 79.7% to 88.7%), productive tiller numbers (from 11.5 to 14.6), and spikelet number per panicle (from 176.7 to 230.2). Notably, all the M628S derived hybrids had higher grain yields than FengLiangyou 4 (SS^HN, Table 4), the control for hybrid variety comparison experiments in the middle and lower reaches of the Yangtze River (Zhou *et al.*, 2007; Song *et al.*, 2021).

Improved performances of hybrids derived from the MC815S

Introgression of N-type *hd1* (Hap2 with a 4-bp deletion) from Minghui 63 to C815S was conducted through four times continuous backcrosses with marker assisted selection for each generation (Supplementary Fig. S3). Our previously designed *Hd1* gene-based InDel marker S56 (Zhang *et al.*, 2015) was used to screen for plants with heterozygous *Hd1/hd1* in each generation under short-day conditions. A male sterile BC_4F_1 plant was selected under LDs in Wuhan and its ratoon was moved to Hainan with short day-length and low temperature growth conditions to generate BC_4F_2 seeds. A male sterile BC_4F_2 plant with homozygous *hd1* was identified as modified C815S (MC815S) in Wuhan. Thus, MC815S maintained genic male sterility under LDs (Fig. 3A). Then, the hybrid with the S^HS^HN combination was obtained by crossing MC815S (NNN) with 9311 (SSN). The modified hybrids (C815SM/9311, S^HS^HN) headed much earlier than the original hybrids (C815S/9311, $S^HS^HF^H$) under natural LDs (Fig. 3B). The modified S^HS^HN hybrids had a heading date of 97.0 d, approximately 42.0 d earlier than that (139.3 d) of the original $S^HS^HF^H$ hybrids (Fig. 3C). Moreover, the grain yield was greatly improved from nothing (extremely late heading causing no filled seeds) for $S^HS^HF^H$ hybrids to 40.2 g for S^HS^HN hybrids (Fig. 3D).

Discussion

Heading date genes greatly contribute to yield heterosis in three-line and two-line hybrid rice

Heading date determines the life cycle of rice plants. Within a reasonable range, late heading provides plants with more time to grow and produce more biomass than early heading does. Thus, high-yield rice cultivars grown in a region should have optimized heading dates to fully take advantage of natural photothermal resources. However, photothermal resources in a given region exhibit some fluctuations across years (Lv *et al.*, 2016). Therefore, the optimized heading date of a hybrid/cultivar must have the plasticity to buffer the risk posed by low temperature in rice production during the grain filling stage. The optimized heading date can be designed by making diverse gene combinations of *Ghd7*, *Ghd8*, and *Hd1* that exhibit large variations in heading date and yield potential in rice (Zhang *et al.*, 2015).

A genome-wide association study was used to identify QTLs contributing to rice grain yield in 1495 elite hybrids (Huang

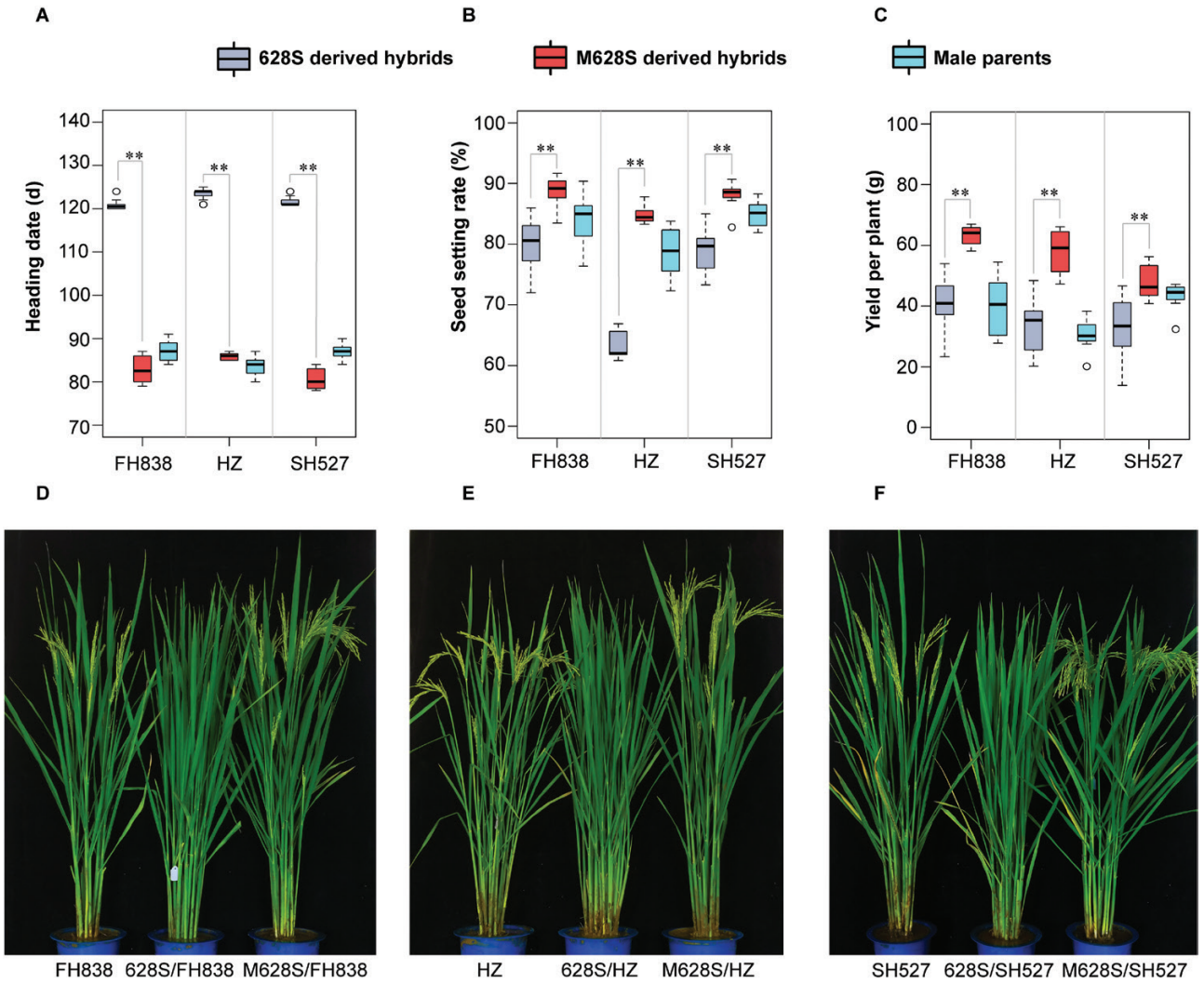

Figure 2. Comparison of heading date, seed setting rate and grain yield per plant between the original and improved hybrids of 628S and M628S under LDs. (A–C) Boxplots of the heading date, seed setting rate, and yield per plant. In each of (A–C), there are three groups, including FH838 (Fuhui 838), HZ (Huazhan), and SH527 (Shuhui 527). For example, for the group of FH838, the 628S derived hybrid was 628S/FH838, the male parent was FH838, and the M628S derived hybrid was M628S/FH838. (D–F) Plants FH838, HZ, SH527, and their hybrids. **Significant difference, $P \leq 0.01$ ($n=16$). 628S is the wild-type, while M628S is the homozygous *hd1* mutant of 628S.

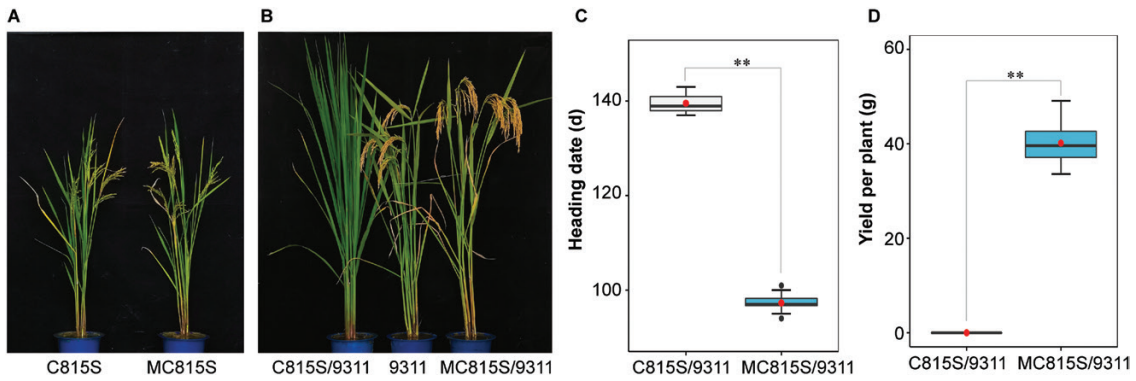

Figure 3. Comparison of agronomic performances between the hybrids produced by original and improved C815S under LDs. (A, B) The field performances of the parents and hybrids. (C, D) Boxplots of heading date and yield per plant. **Significant difference, $P \leq 0.01$ ($n=16$). C815S is the wild-type, while MC815S is the modified line of C815S with N-type *Hd1*.

et al., 2015). It revealed that the flowering genes *OsSOC1*, *Ghd7*, and *Ghd8* had the largest effects on grain yield per plant, and *Hd1* had extremely high heterozygosity. Then, 10 074 F_2 plants derived from 17 of the 1495 hybrids were used for the identification of heterotic QTLs with effects on grain yield (Huang *et al.*, 2016). Interestingly again, flowering genes were closely associated with yield heterosis. For example, *Hd3a* had a strong effect on grain yield in three-line hybrids, *Ghd8* was an important contributor in two-line hybrids, and *OsGI* and *Hd1* had large effects on grain yield in *indica–japonica* hybrids. Moreover, *Ghd7* was highly differentiated between restorer and CMS lines in three-line hybrids. In addition, *Ghd8* was found to be the major gene contributing to yield heterosis in two-line hybrids in the heterozygous state (Li *et al.*, 2016). The flowering genes *Ghd7*, *Hd3a*, *Ehd2*, and *Ehd4* were demonstrated to be highly differentiated and associated with yield heterosis in superior three-line hybrids, and the flowering gene *OsCOL4* was associated in superior two-line hybrids in a recent study (Lv *et al.*, 2020). Lately, flowering genes, including *Ghd8*, were once again proved to be differentially introgressed in parents to shape heterotic loci in the hybrid rice (Lin *et al.*, 2020). Notably, *Ghd7* was not associated in two-line hybrids (Huang *et al.*, 2016) while *Ghd8* was not associated in three-line hybrids by genome-wide association study (Lv *et al.*, 2020). The failed identification of *Ghd7* is caused by its uniform function in the populations in which all six investigated two-line hybrids carry homozygous S-type *Ghd7* alleles except that one female parent Y58S carries W-type *Ghd7* according to our gene diagnosis here (Supplementary Table S8). But *Ghd7* made important contributions to hybrid performance because homozygous S-type alleles in both parents contribute a higher mid-parent value than homozygous N-type alleles in both parents or S-type alleles in one parent and N-type alleles in another parent. In contrast, the failed identification of *Ghd8* was probably caused by its lack of contribution to the performance of the investigated hybrids in which no or rare *Ghd8* alleles are functional. This explanation is supported by this study, in which there are only a very few three-line hybrids derived from HL-CMS Yuetai A and IP-CMS Quan 9311A that carry S-type *Ghd8* alleles in the middle and lower reaches of Yangtze River Basin (Supplementary Table S5).

Flowering genes are frequently associated with yield and heterosis in several large rice collections (Li *et al.*, 2016; Huang *et al.*, 2016; Lin *et al.*, 2020; Lv *et al.*, 2020), indicating that heading date is closely related to hybrid yield and heterosis. The first three-line hybrid rice, Nanyou 2, was developed in 1974 (Li and Xin, 2000). Subsequently, Chinese hybrid rice has undergone six rounds of variety renovation (Zeng, 2018). Comparison of major representative hybrids across the six rounds showed that most hybrids possess the S_NF_ (main for three-line hybrids) or S_S_N (main for two-line hybrids) combinations (Supplementary Table S9). This probably indicated that appropriate heading dates are required for strong heterosis, and these main combinations are always maintained by breeders during hybrid renovation. On the other hand, other agronomic traits, especially for disease resistance and quality improvement, are likely the major causes for hybrid renovation.

Dominant three-gene combinations for hybrids with strong heterosis

The S^HNF^H combination is most common in three-line hybrids (Supplementary Table S5). The typical representative hybrid Shanyou 63 had been ever planted in half of China, covering a total of approximately 63 million hectares from 1982 to 2019 (Xie and Zhang, 2018) (http://www.ricedata.cn/variety/). The photoperiod sensitivity of Shanyou 63 (S^HNF^H) is not very strong, ensuring its widespread adaptability (Xie and Zhang, 2018). It was approved for commercial rice production in 16 provinces of central and southern China, from 18°N (Hainan) to 38°N (Shandong) geographically (Xie and Zhang, 2018). In the early stage of three-line hybrid production, the contribution of *Ghd8* to heterosis is very limited because the F-type *Hd1* in WA-CMS parents and the S-type *Ghd7* in the corresponding restorer parents excluded the introgression of *Ghd8* (Tables 1, 3).

The S_S_N hybrids (SSN, SS^HN, S^HSN, and S^HS^HN) are predominant for strong yield heterosis in two-line hybrids (Supplementary Table S5). The SSN combination has very strong photoperiod sensitivity and exhibits late heading (Zhang *et al.*, 2015). The varieties with the SSN combination head 9.6 d later than those with the SNF combination on average in a germplasm population of 328 inbred varieties (Supplementary Table S10). Accordingly, the plants with the SSN combination headed 10.6 d later than those with SNF and had a higher yield in an F_2 population derived from Zhenshan 97/9311 (Supplementary Table S10). Moreover, in the NIL-F_2 population with the background of Zhenshan 97, the plants with the S_S_N combinations headed 16.7 d later than those with the S_NF_ combinations on average (Supplementary Table S10). In summary, plants with the S_S_N combinations exhibit vegetative growth approximately 10.0 d longer than those with the S_NF_ combinations, which increases the grain yield potential. This is one of the reasons why two-line hybrids (S_S_N in common) often produce a higher yield than three-line hybrids (S_NF_ in common) as previously reported (Li and Yuan, 2000; Yang *et al.*, 2009; Jiang *et al.*, 2015; Wu *et al.*, 2016; Zhen *et al.*, 2017; Wu *et al.*, 2018). This is also the main reason why *Hd1* was chosen to be knocked out in 628S (NSF) to generate M628S (NSN), retaining the strong heterosis effect of *Ghd8* in this study (Figs 1, 2). These modifications can resolve the problem wherein approximately half of over 800 crosses produced by 628S flowered too late to bear filled grains in the middle and lower reaches of Yangtze River in a previous study (Fu *et al.*, 2010).

The S_S_N combinations of three-line hybrids were identified in only a few of the ones produced by the limited HL-CMS

lines and their restorer lines in the middle and lower reaches of Yangtze River (Supplementary Table S5). Recently, the newly developed commercial IP-CMS line Quan 9311B (A) with an NSN combination was used to produce $S^{H}S^{H}N$ hybrids such as the elite hybrid rice varieties QuanyouHuazhan and QuanyouSimiao. Hybrids with S_S_N combinations have not yet been widely expanded in the three-line system. It is strongly suggested that replacement of *Hd1* with *Ghd8* will greatly contribute to heterosis in three-line hybrids of the middle and lower reaches of the Yangtze River. Taken together, for optimization of yield production, the S_NF_ combinations are suggested to be the ideal genotypes in ecological regions around 30°N/S, including East and South Asia (mainly in Central China, Pakistan, and India), Australia, Africa (e.g. Egypt), South America (such as Uruguay and Argentina) and North America (USA), and the S_S_N combinations are favorable genotypes in lower latitude areas around 23.5°N/S mainly in southern China of East Asia, South-East and South Asia (such as Pakistan, India, Bangladesh, Myanmar, and Philippines), Australia, Africa (such as Madagascar, Nigeria, Egypt), South America (mainly in Brazil and Argentina) rich in photothermal resources (Supplementary Fig. S4). For instance, the $SS^{H}N$ combination hybrid rice Honglianyou 6 exhibited high yield in countries along the 'One Belt One Road' such as South-East and South Asian and African countries (Zhu, 2016). However, hybrids with S_S_F_ combinations were not found in Chinese commercial hybrids in the middle and lower reaches of the Yangtze River because they have extremely strong photoperiod sensitivities and very late heading dates. These hybrids are suitable for growth in tropical regions (such as parts of Africa, South-East and South Asian, and South American countries) that possess short day lengths and warm temperatures throughout the year, which provides the chance to show yield potential (Zhang *et al.*, 2015) (Supplementary Fig. S4). As reported, loss of floral repressor function adapts rice to higher latitudes (Jorge *et al.*, 2015), and thus the S_NN combinations or others with lower photoperiod sensitivities are suitable for higher latitude regions around 38° or as double cropping late rice in lower latitude areas (Supplementary Fig. S4).

Synchronizing the heading date of parents to produce more hybrids by breeding design

Developing hybrid seeds is an important step in hybrid rice production. Synchronizing the heading date between parents is a prerequisite for developing high yield hybrid seeds (Mao *et al.*, 1998). In most cases, there are large differences in heading date between male and female parents. Usually, male parents head much later than female parents in the three-line hybrid system. For example, the parents of the super hybrid Shanyou 63 have a difference of approximately 28.0 d in heading date (Zhenshan 97A has a heading date of approximately 62.0 d, while that of Minghui 63 is approximately 90.4 d; Supplementary Table S11). Synchronizing the heading date of parents by staggered sowing is a widely used method for developing hybrid seeds, but it is risky because the temperature frequently fluctuates during the rice growing season. Breeding of heading date synchronized parents is an easy way to develop more hybrid seeds.

To synchronize heading date, flowering genes can be allocated to the parents once the optimized combinations are defined to a region. For example, the newly developed $ZS97^{Ghd7}$ (Zhenshan 97 introgressed with S-type *Ghd7*, SNF) has a heading date of 87.0 d, Minghui 63 (SNN) has a heading date of approximately 90.0 d, and the performance of the modified hybrid Shanyou 63 (SNF^{H}) between $ZS97^{Ghd7}$ and Minghui 63 is similar to that of the original Shanyou 63 ($S^{H}NF^{H}$), but it is easy to synchronize the heading date of parents with a small difference, which ensures the production of more hybrid seeds (Supplementary Table S11). Homozygous and heterozygous combinations showed a few days' difference in heading date (Supplementary Table S12). The hybrids of S_NF_ combinations can be generated by several parental combinations, and parents with a small difference of less than 7.0 d in heading date can be developed to produce more hybrids (Supplementary Table S12). Accordingly, for easier flowering synchrony, hybrids with S_S_N combinations can be produced by crossing a female parent NSN with a male parent SNN, or crossing a female parent SSN with a male parent SSN (Supplementary Table S12). On the one hand, S-type *Ghd8* has been reported as the dominant gene for heterosis exploration in two-line

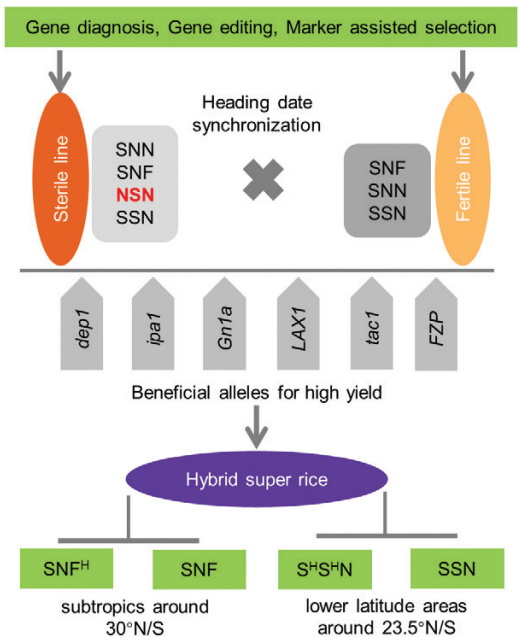

Figure 4. A model for breeding heading date synchronized parents to generate hybrid super rice. Sterile and fertile lines were designed to have three-gene combinations of *Ghd7*, *Ghd8*, and *Hd1* suitable for synchronizing the heading date of parents to generate hybrid super rice. Combinations with three uppercase letters represent functional classifications of *Ghd7*, *Ghd8*, and *Hd1*. The NSN (sequential three-gene combination of N-type *Ghd7*, S-type *Ghd8*, and N-type *Hd1*) combination is highly recommended as the ideal combination for female lines.

hybrids (Li *et al.*, 2016; Huang *et al.*, 2016), and the SNN combination is common in restorer lines (Table 3). On the other hand, parents with NSN and SNN combinations are expected to have very similar heading dates (Supplementary Table S12). Consequently, more $S^{H}S^{H}N$ hybrid seeds would be easily developed by crossing both parents (Supplementary Table S12). Thus, the combination NSN is highly recommended as the ideal combination for female parents in practices involving two-line and three-line hybrids in China, because just 1.6% (3 out of 190) of investigated parents have this combination (Supplementary Table S3). Considering heading date genes and yield related genes, a model for hybrid super rice breeding was proposed (Fig. 4). Once the heading date genes are defined, other favorable alleles of yield related genes are included to develop the parents for further enhancing yield in hybrids. For example, *tac1* (Yu *et al.*, 2007) and *ipa1/osspl14* (Jiao *et al.*, 2010; Miura *et al.*, 2010) for plant architecture, *Gn1a* (Ashikari *et al.*, 2005), *LAX1* (Komatsu *et al.*, 2001), *DEP1* (Huang *et al.*, 2009), and *FZP* (Bai *et al.*, 2017) for inflorescence architecture, and *nal1* (Fujita *et al.*, 2013) for spikelet number are targets for enhancing yield. Hence, these beneficial alleles of yield related genes could be constantly utlized in newly released hybrids.

Supplementary data

The following supplementary data are available at *JXB* online.

Fig. S1. Haplotypes of *Ghd7*, *Ghd8*, and *Hd1* included in this study.

Fig. S2. 'Lao-lai-qing' plants collected in the fields

Fig. S3. Breeding scheme for substitution of *hd1* with *Hd1* in C815S.

Fig. S4. The suggested three-gene combination hybrids with optimized yield potentials for diverse planting areas.

Table S1. The primers used in this study.

Table S2. Function analysis of *Ghd7* Hap2.

Table S3. Summary of the combinations in hybrid parents included in this study.

Table S4. Kinds of S_S_F_ hybrids generated in theory.

Table S5. The list of hybrids whose parents were sequenced in the present study.

Table S6. Gene diagnosis for eight 'Lao-lai-qing' plants with the three genes.

Table S7. Gene diagnosis of 628S, C815S, and the restorer lines.

Table S8. Three-gene combinations of the six two-line hybrids used in previous study.

Table S9. Renovation of hybrid rice varieties in China.

Table S10. Comparison of heading date and grain yield between the S_S_N and S_NF_ combinations in three populations.

Table S11. Performances of the original and modified Shanyou 63 hybrids and their parents with allocated flowering genes.

Table S12. Comparison of heading date between homozygous and heterozygous combinations, and recommended ideal combinations for flowering synchrony in hybrid parents with allocated flowering genes.

Acknowledgements

We would like to thank Prof. Yaoguang Liu (South China Agriculture University, China) for providing the CRISPR/Cas9 vectors and Mr Jianbo Wang for his excellent work in the paddy field.

Author contributions

XZ conducted most of the lab and field experiments and collected and analysed data. CN generated M628S and the corresponding hybrids. BW contributed in the improvement of C815S. TZ developed NIL $ZS97^{Ghd7}$ line, Shanyou 63 and modified Shanyou 63 hybrids. BZ developed the NIL-F_2 population and collected the heading date trait. XL conducted gene diagnosis of the parental lines. GG, JM, QZ, HL, SL, ZL, YH, TM, SG, SQ, and YY sampled the collection of parental lines. QZ made suggestions for writing. YX and XZ designed and supervised the experiments and data analysis, and wrote the manuscript.

Conflict of interest

The authors declare that they have no conflicts of interest.

Funding

This work was supported by grants from National Key Research and Development Program of China (2016YFD0100802), National Natural Science Foundation of China-NSFC-ASRT (Chinese-Egyptian) Cooperative Research project (32061143042) and National Natural Science Foundation of China (91935302, 31821005, 31901519).

Data availability

All data supporting the findings of this study are available within the paper and within its supplementary data published online.

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