Ef-cd locus shortens rice maturity duration without yield penalty

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The contradiction between "high yielding" and "early maturing" hampers further improvement of annual rice yield. Here we report the positional cloning of a major maturity duration regulatory gene, *Early flowering-completely dominant* (*Ef-cd*), and demonstrate that natural variation in *Ef-cd* could be used to overcome the above contradictory. The *Ef-cd* locus gives rise to a long noncoding RNA (lncRNA) antisense transcript overlapping the *OsSOC1* gene. *Ef-cd* lncRNA expression positively correlates with the expression of *OsSOC1* and H3K36me3 deposition. Field test comparisons of early maturing *Ef-cd* near-isogenic lines with their wild types as well as of the derivative early maturing hybrids with their wild-type hybrids conducted under different latitudes determined that the early maturing *Ef-cd* allele shortens maturity duration (ranging from 7 to 20 d) without a concomitant yield penalty. *Ef-cd* facilitates nitrogen utilization and also improves the photosynthesis rate. Analysis of 1,439 elite hybrid rice varieties revealed that the 16 homozygotes and 299 heterozygotes possessing *Ef-cd* matured significantly earlier. Therefore, *Ef-cd* could be a vital contributor of elite early maturing hybrid varieties in balancing grain yield with maturity duration.

lncRNA | rice | heading date | yield

The growing world population calls for continued increases in food production (1). Elevating grain yield per unit area is one of the most effective ways to increase food production because urbanization continues to decrease the area available for growing crops (2). Normally, a crop plant which produces higher yield needs a longer growth period because the production rate of carbohydrate via photosynthesis is certainly restricted (3, 4). In other words, early maturing cultivars usually have a low yield, suffering from shortening maturity duration in comparison with late-maturing cultivars.

The first generation of green revolution semi-dwarf varieties, such as IR8, IR20, IR24, and IR26, successfully provided large yield increases; however, these varieties required up to 160 d or longer for maturing or harvesting (5, 6). Likewise, Shanyou1 and Shanyou2, the first generation of hybrid cultivars commercialized in the late 1970s in China, also offer notable yield increase, but are again characterized as having a slow harvest turnaround time (5, 7, 8). In rice breeding, "high yielding" and "early maturing" remains a long-standing paradox, and the development of early maturing and high-yielding cultivars has been a great challenge.

Rice breeders have invested much effort to develop early maturing cultivars that allow for multiple crops per year. Ce64 is such an early maturing restorer line, and its derivative hybrids, Weiyou64 and Shanyou64, were the first 2 hybrid cultivars that could produce 2 crops a year (9). Subsequently, another early maturing restorer line, Minghui77, has played a leading role, demonstrated by 7,446,700 ha covered by its derivative hybrids from 1991 to 2010 in China (10). Similarly, the International Rice Research Institute (IRRI) released the earlier maturing IR36 with 110 d of maturity in 1976 and subsequently released IR50 with 105 d and IR58 with 100 d (6). Until now, although many related genes have been cloned and functionally characterized (11, 12), it is still difficult to overcome the negative association that causes early maturing cultivars to suffer a yield penalty from shortened vegetative growth periods. Here, we addressed this by cloning the dominant *Early flowering-completely dominant* (*Ef-cd*) gene and found that the *Ef-cd* locus significantly

Significance

Early maturing rice cultivars usually have a low yield, suffering from a shortening maturity duration in comparison with late-maturing ones. Here we demonstrate that shortening of maturity duration with no yield penalty of rice can be achieved by the quantitative trait locus *Early flowering-completely dominant* (*Ef-cd*). *Ef-cd* is a long noncoding RNA transcribed from the antisense strand of the flowering activator *OsSOC1* locus, which can positively regulate the expression of *OsSOC1*. Physiological analysis demonstrated that *Ef-cd* could facilitate nitrogen utilization and also improve the photosynthesis rate. This study provides breeders with a valuable genetic resource that is useful for balancing grain yield with maturity duration for further elevating food production in the world.

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shortens maturity durations to around 7 to 20 d in near-isogenic lines (NILs) and hybrid cultivars without causing a yield penalty.

Ef-cd, first discovered from the male sterile line 6442S-7 as a dominant locus for earliness, has been mapped to the short arm on chromosome 3 (13, 14). Then *Ef-cd* was introgressed into 6 late-maturing cultivars, creating a set of 6 high-generation NILs for *Ef-cd* (ranging from BC_5 to BC_{13}, and F_9 to F_{14}) (*SI Appendix*, Fig. S1*A*). When tested under 3 different photoperiod environments across China, the NILs headed consistently earlier than their recurrent parents, flowering on average 8.9 to 19.7 d earlier depending on geographical location (*SI Appendix*, Fig. S1*B*).

To identify the underlying gene for *Ef-cd*, we conducted high-resolution mapping using 4,800 F_2 late-maturing plants derived from a cross between early maturing NIL D248 (BC_5F_{11}) and its recurrent parent Shuhui881 (SH881) (Fig. 1*A*). Finally, we fine-mapped *Ef-cd* in a 12.9-kb interval flanked by InDel4-3 and InDel5-2 (Fig. 1*B*). The marker InDel5-2 was located in the first intron of *Os03g0122600*, which indicated that *Os03g0122500* and part of *Os03g0122600* was located in the mapped region (*SI Appendix*, Fig. S2). *Os03g0122600*, also known as *OsSOC1*/*OsMADS50*/*DTH3*, was previously reported as a flowering activator (15, 16). *Os03g0122500* is transcribed from the antisense strand of *Os03g0122600*, and its transcription is supported by a full-length cDNA AK242050 that is annotated as a long noncoding RNA (lncRNA) (https://rapdb.dna.affrc.go.jp). Compared with SH881, D248 harbors a number of genetic variations in the promoter region, as well as 1 insertion in the first intron and 1 single-nucleotide polymorphism in the second intron of *Os03g0122500* (*SI Appendix*, Fig. S2).

It is a common challenge to disentangle the individual effects of sense and antisense transcripts, as altered antisense transcript may simultaneously affect the expression of sense transcript (17–19). Similar to the *Ef-cd* transcribed from the antisense strand of the *OsSOC1* locus, in humans, a lncRNA *HOTTIP* is transcribed from the *HOXA* locus, and ectopic expression of *HOTTIP* RNA failed to activate expression of the distal *HOXA* gene, suggesting that the precise genomic distance between lncRNA and its target gene is critical for the colinear activation (20). Since the entire *OsSOC1* spans nearly 30 kb in the rice genome, and also the early maturing NILs and their recurrent parents are *indica* varieties which are recalcitrant to transform, it is difficult to perform complementation experiments with constructs containing *Ef-cd* or *OsSOC1*. To verify that *Os03g0122500* but not *OsSOC1* was *Ef-cd*, we screened T-DNA libraries and luckily found 2 ingenious T-DNA insertion mutant lines, one (*ef-cd-1*) with a insertion in the first intron of *Os03g0122500* and the other (*ef-cd-2*) with an insertion in the promoter region of *Os03g0122500* but away from the 3′UTR of *OsSOC1*. As a result of the lesions in

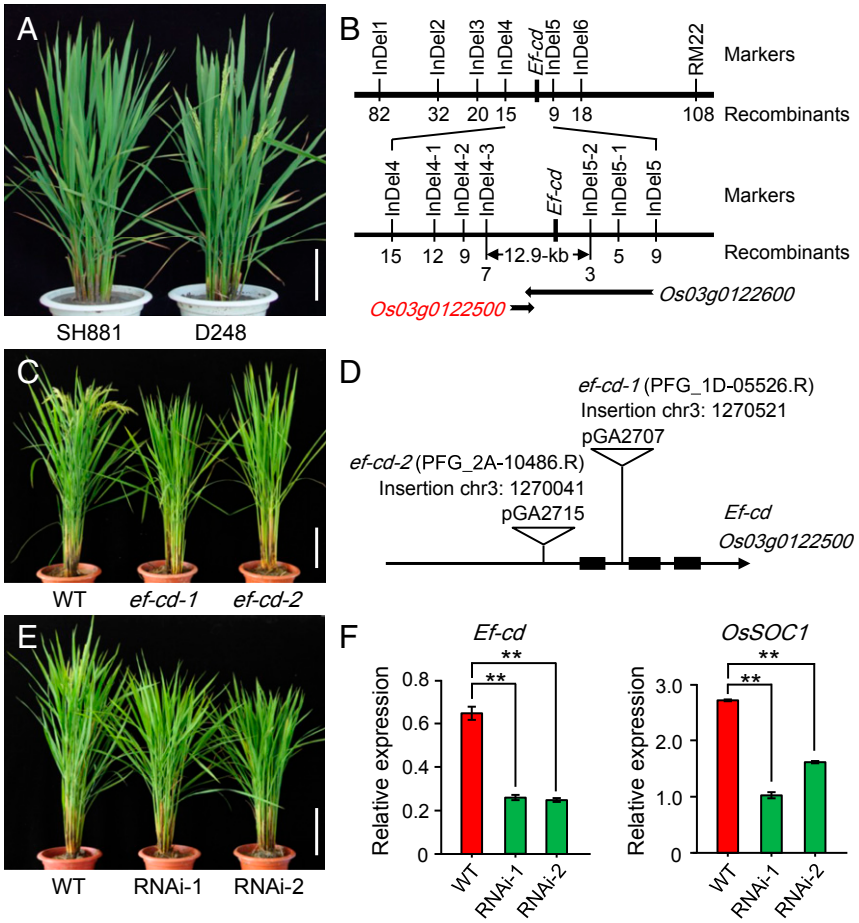

Fig. 1. *Os03g0122500* was the causal gene of *Ef-cd*. (*A*) NIL D248 headed earlier than its recurrent parent Shuhui881 (SH881). (*B*) *Ef-cd* was mapped in a 12.9-kb region. (*C*) Two T-DNA insertion mutants of *Os03g0122500* headed later than the wild-type Hwayoung. (*D*) The insertion occurred in the first intron of *Os03g0122500* in mutant *ef-cd-1* and in the promoter region of *Os03g0122500* in mutant *ef-cd-2*. The triangle represents the T-DNA insertion. The types of the binary vectors used for constructing the T-DNA insertion mutants are above the triangles. (*E*) Two RNAi lines of *Os03g0122500* headed later than their wild-type Nipponbare. (*F*) Expression levels of *Ef-cd* and *OsSOC1* were significantly decreased in the RNAi lines compared to their wild type. The *P* value was calculated using Student's *t* test. $^{**}P < 0.01$. (Scale bars, 20 cm.)

Os03g0122500, both T-DNA lines headed later than the wild type (Fig. 1 *C* and *D*). We also generated a mutant harboring a 158-bp deletion in the promoter of *Os03g0122500* by using CRISPR/Cas9 technology, and the mutant also showed an expected later flowering phenotype (*SI Appendix*, Fig. S3 *A*–*C*). Furthermore, a fragment in the first exon of *Os03g0122500* was used to generate customized RNA interference (RNAi) transgenic plants for *Os03g0122500*. All of the RNAi transgenic lines with reduced expression of *Os03g0122500* showed a later flowering phenotype (Fig. 1 *E* and *F*). As there was not any variant in the *Os03g0122500*-coding region between NIL D248 and SH881, we hypothesized that the variants in its promoter region might affect its transcription. To this end, we fused the promoter fragments from NIL D248 and its recurrent parent SH881, respectively, with the firefly luciferase-coding sequence and assayed the activity in vitro. The luciferase activity of *pD248::LUC* was 7.34 times higher than that of *pSH881::LUC* (*SI Appendix*, Fig. S3*D*), indicating that the promoter of *Os03g0122500* in D248 has much higher activity. All of the above results cumulatively indicate that *Os03g0122500* is *Ef-cd*, as nucleotide variants in the promoter of *Os03g0122500* modulated its transcriptional activity and contributed to the phenotypic consequences.

Because the floral transition of wild-type plants (SH881) generally occurred at about the 60th day after germination in both long-day and short-day conditions, we checked *Ef-cd* and *OsSOC1* expression levels before this stage. The results showed stronger expression of *Ef-cd* and *OsSOC1* in the early maturing NIL D248 than in its wild type, and the expression levels of *Ef-cd* and *OsSOC1* were positively correlated (*SI Appendix*, Fig. S4 *A* and *B*). Furthermore, the *OsSOC1* expression levels were significantly reduced in the *ef-cd* T-DNA mutants (*SI Appendix*, Fig. S4 *C* and *D*). In contrast, the *Ef-cd* expression level was not significantly reduced in the *Ossoc1* mutant plants (*SI Appendix*, Fig. S4 *E* and *F*). These results suggest that *Ef-cd* regulates the expression of *OsSOC1*, but not vice versa. In addition, the expression levels of both *Hd3a* and *RFT1* (2 rice florigen genes) were significantly higher in D248 than those in SH881 in both short-day and long-day conditions (*SI Appendix*, Fig. S4 *G* and *H*).

Field tests revealed that, except for plant height, there were no statistical differences between early maturing NILs and their late-maturing recurrent parents for the yield-related agronomic traits, demonstrating no yield penalty from *Ef-cd* (Dataset S1). Shanyou63 (SY63) is a hybrid cultivar with the largest planting area in China, as is II-You838 in southern China. SY63 is derived from the cross between the male-sterile line Zhenshan97A (ZS97A) and the restorer line Minghui63 (MH63), and II-You838 is derived from the cross between the male-sterile line II-32A and the restorer line Fuhui838 (FH838). Using NILs containing the early maturing *Ef-cd* allele in the MH63 (named D330) or II-32A (named E-II-32A) background, we developed F_1 hybrids derived from crosses between ZS97A and D330 (named E-SY63) and from crosses between E-II-32A and FH838 (named E-II-You838). Then we conducted pairwise field testing of E-SY63 (ZS97A/D330) with SY63 (ZS97A/MH63) and of E-II-You838 (E-II-32A/FH838) with II-You838 (II-32A/FH838) in 4 different locations across China. Both of the *Ef-cd* NIL hybrids matured earlier than their corresponding late-maturing hybrids in all 4 locations (Fig. 2 *A* and *B*). More interestingly, the 2 NIL hybrids produced greater yields than their corresponding hybrids in Beijing (Fig. 2*C*), and 1 NIL hybrid produced a greater yield in Jiaxing (Fig. 2*D*), while yields did not differ in the other 2 locations (Fig. 2 *E* and *F*). These results strongly demonstrate no yield penalty in hybrids that matured earlier due to introgression of the *Ef-cd* locus into the parental male-sterile lines and/or restorer lines of hybrid rice. It should be noted that the 2 NIL hybrids headed earlier in a range from 6.9 to 15.9 d in 4 different locations, suggesting that *Ef-cd* might also coordinate with the environmental factors, such as temperature and day length, in addition to genetic background.

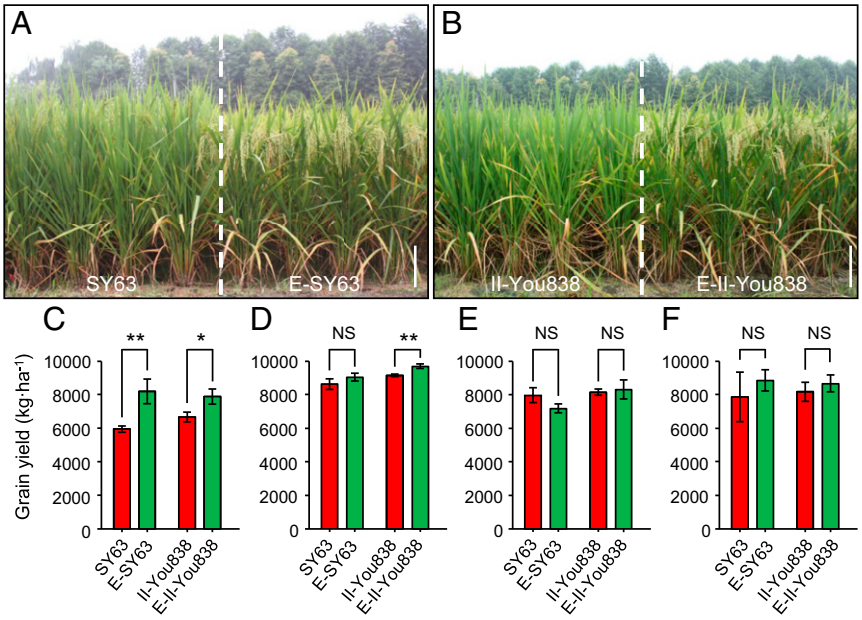

Fig. 2. Analysis of heading date and grain yield for the effect of *Ef-cd* in 4 different latitudes including Beijing (39°54′ N, Beijing City), Jiaxing (30°75′ N, Zhejiang Province), Chengdu (30°42′ N, Sichuan Province), and Fuzhou (26°08′ N, Fujian Province). (*A*) *Ef-cd* NIL hybrid E-SY63 (ZS97A/D330) headed and matured earlier than the corresponding hybrid SY63 (ZS97A/MH63) in the 4 different latitudes. (*B*) *Ef-cd* NIL hybrid E-II-You838 (E-II-32A/FH838) headed and matured earlier than the corresponding hybrid II-You838 (II-32A/FH838) in the 4 different latitudes. Statistical comparison of the grain yields between SY63 and E-SY63 and II-You838 and E-II-You838 in (*C*) Beijing (sowing date was May 8, 2015), (*D*) Jiaxing (sowing date was June 30, 2015), (*E*) Chengdu (sowing date was May 15, 2015), and (*F*) Fuzhou (sowing date was June 18, 2015). The *P* value was calculated using Student's *t* test. $*P < 0.05$; $**P < 0.01$; NS, not significant. (Scale bars, 20 cm.)

To explore its physiological basis, we conducted ^{15}N-nitrate and ^{15}N-ammonium feeding experiments with the early maturing NIL and its recurrent parent and found that the acquisition of nitrate and ammonium increased significantly more in the early maturing NIL than in the recurrent parent (*SI Appendix*, Fig. S5). In addition, we also measured the physiological features of E-SY63 and SY63, and the results showed that the leaf length, leaf width, leaf area index, chlorophyll concentration, and light-saturated photosynthetic rate were all significantly increased in E-SY63 compared with those in SY63 (*SI Appendix*, Fig. S6). Furthermore, the strand-specific RNA sequencing (ssRNA-seq) was carried out, and differentially expressed genes (DEGs) between the early maturing NIL D248 and its recurrent parent SH881 were analyzed. In total, 2,075 DEGs were identified, and 782 of them showed up-regulation and 1,293 of them showed down-regulation in the early maturing NIL (Dataset S2). According to Gene Ontology (GO) enrichment analysis, the "cellular nitrogen compound metabolic process" and the "chlorophyll metabolic process" were in the top 5 enriched GO terms of the up-regulated genes (*SI Appendix*, Fig. S7*A*), so we investigated the DEGs which related to nitrogen metabolism, chlorophyll metabolism, and photosynthesis. We found 16 DEGs related to nitrogen metabolism, and 11 of them showed up-regulation in the early maturing NIL (*SI Appendix*, Fig. S7*B*). In addition, 18 DEGs were related to chlorophyll metabolism and photosynthesis, and 15 of them showed up-regulation in the early maturing NIL (*SI Appendix*, Fig. S7*C*). Recently, yield potential of crops has been considered to be limited by photosynthesis (21, 22) and photosynthetic capacity closely related to leaf N content in C_3 plants (23, 24). In our study, many of the DEGs had been reported to facilitate nitrogen utilization (25–31) and improve photosynthesis (32–35). These results provided further supportive evidence of the regulatory role of *Ef-cd* in shortening rice maturity duration without yield penalty. Of course, plant life span is complicated, and additional factors may be involved.

Natural antisense transcripts can exert their regulatory functions by acting as epigenetic regulators of gene expression and chromatin modifications (20, 36–39). Epigenome data showed that the chromatin modifications were obvious around the *Ef-cd-OsSOC1* locus (http://structuralbiology.cau.edu.cn/cgi-bin/hgTracks) (*SI Appendix*, Fig. S8*A*). Histone Lys methylation is a well-studied epigenetic modification with both activating and repressing roles in gene expression (40). In our previous study, the level of H3K36me2/3 in the *OsSOC1* chromatin region was decreased in the late-flowering mutant *lvp1*/*sdg724* (41). In this study, we compared 3 mutants with their wild types for the expression levels of both *Ef-cd* and *OsSOC1*: the *sdg724* mutant with a modification of H3K36me2/3 (41); *lc2* as a component of the PRC2 complex mutant with a modification of H3K27me2/3 (42); and *chr729* as a subunit of the chromatin-remodeling complexes with a modification of H3K4me2/3 (43). Except for *lc2* mutant, the expressions of *Ef-cd* and *OsSOC1* decreased significantly in both late-flowering mutants *chr729* and *sdg724* (*SI Appendix*, Fig. S8 *B–D*). These results indicated that the *Ef-cd-OsSOC1* locus had complex chromatin modifications. Meanwhile, using SH881 and NIL D248, we investigated the H3K36me3 level at 5 loci within the *OsSOC1* gene (Fig. 3*A*) and found that the level of H3K36me3 increased in D248 at the second, third, and fourth loci (Fig. 3*B*). Additionally, The results of chromatin immunoprecipitation sequencing (ChIP-seq) and ssRNA-seq further confirmed that D248 had a higher level of H3K36me3 and a higher expression level surrounding the *OsSOC1* locus compared with SH881 (Fig. 3*C*). Recently, an increasing number of studies have revealed that the internal transcription initiation occurs frequently in the genes of eukaryotes (44, 45). In our study, the results of ssRNA-seq forward strand showed that other natural antisense transcripts (NATs) could exist in the long intron of *OsSOC1* (Fig. 3*C*). Transcript structure analysis by StringTie also demonstrated that, in addition to *Os03g0122500-1*, other isoforms may exist for *Ef-cd* (*SI Appendix*, Fig. S9*A*). Many isoforms of *Ef-cd* with polyadenylated tails were also detected by a 3′ rapid amplification of cDNA ends (3′-RACE) assay (*SI Appendix*, Fig. S9 *B* and *C*). Moreover, the DNaseI track revealed an obvious DNaseI peak in the long intron of *OsSOC1*, which implied that alternative transcript isoforms of *OsSOC1* could exist

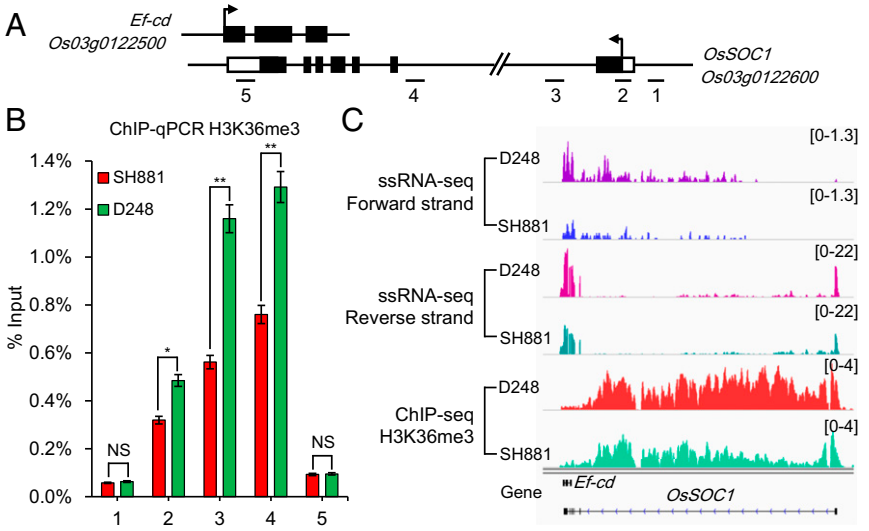

Fig. 3. Analysis of H3K36me3 level and mRNA expression level. (*A*) Genomic structure of the *Ef-cd* and *OsSOC1* locus. Black solid boxes indicate exons, and white boxes indicate untranslated regions. The number under each line corresponds to the number on the *x* axis in B for the H3K36me3 level in the region. (*B*) The results of ChIP-qPCR analysis to confirm H3K36me3-binding sites in SH881 and D248 surrounding the *OsSOC1* locus. The percentage of ChIP'ed DNA to input DNA (% input) was detected using qPCR of ChIP samples. (*C*) Genomic tracks display gene expression change and H3K36me3 ChIP-seq change in D248 and SH881 surrounding the *Ef-cd* and *OsSOC1* locus. The top 2 tracks are normalized *Ef-cd* intensity; the third and fourth tracks are normalized *OsSOC1* gene intensity; the bottom 2 tracks are normalized genomic coverage of H3K36me3 in D248 and SH881. The *Ef-cd* and *OsSOC1* genomic tracks are shown below the profiles. The *P* value was calculated using Student's *t* test. $*P < 0.05$; $**P < 0.01$; NS, not significant.

and initiate near this intronic DNaseI hypersensitive site (*SI Appendix*, Fig. S8*A*). These NATs and alternative transcript isoforms of *OsSOC1* and *Ef-cd* might also play important roles in phenotypic determination. These features of *Ef-cd* were quite similar to the lncRNA *COOLAIR* described previously (36, 46), and a recent study reported *MAS*, a NAT-lncRNA produced from the *MAF4* locus (47). *MAS* activates *MAF4* by interacting with WDR5a, one core component of the COMPASS-like complexes, and recruiting WDR5a to *MAF4* to enhance H3K4me3 (47). Based on our data, we speculated that *Ef-cd* could recruit an undefined complex that may contain SDG724, which leads to an increase of the H3K36me3 level in the *OsSOC1* locus and promotes the expression of *OsSOC1* (*SI Appendix*, Fig. S10). Further efforts to identify *Ef-cd*–interacting proteins will be of particular importance to obtain new insight into its regulatory mechanism.

According to the pedigree information, the *Ef-cd* locus of D248 was derived from the IRRI variety IR9761-19 (*SI Appendix*, Fig. S11*A*). Therefore, we checked the frequency of the *Ef-cd* locus in rice cultivars using the 122500InDel-1 marker developed by the 36-bp insertion-deletion polymorphism in the promoter sequence of *Ef-cd* between D248 and SH881. All of the early maturing cultivars (Ce64, IR30, IR36, IR50, IR58, IR64, and IR74) were found to contain the *Ef-cd* allele (*SI Appendix*, Fig. S11*B*). Interestingly, the *Ef-cd* locus is common to some Chinese leading early maturing restorer cultivars such as Ce64-7, Minghui77, R402, and To463, but is absent in the leading late-maturing Chinese restorer cultivars such as MH63, 9311, SH881, and Fuhui838 (*SI Appendix*, Fig. S11*C*). These results indicate that *Ef-cd* had clearly been unintentionally exploited in early maturing hybrid rice breeding programs in China. Given the popularity of these restorer lines, the genotypic variation at the *Ef-cd* locus might have contributed to the variance in maturation times among hybrid cultivars. A recent survey among 1,495 elite hybrid rice cultivars via genomic analysis concluded that the *Ef-cd-OsSOC1* locus is the major QTL underlying heading variation in both Sanya and Hangzhou (48). We analyzed 1,439 elite hybrid rice cultivars using the 122500InDel-1 and sf0301285586 markers (49) that were completely linked with the *Ef-cd-OsSOC1* locus (Dataset S3). All of the hybrid cultivars with homozygous and heterozygous *Ef-cd* genotype matured significantly earlier than those with homozygotes of *ef-cd* in both Sanya and Hangzhou (Fig. 4 *A* and *B*). These elite early maturing hybrids have been widely applied for many years in China, demonstrating the vital contribution of the *Ef-cd-OsSOC1* locus to hybrid rice production.

Recently, in China, a breakthrough in yield potential has been achieved through the development of *indica-japonica* intersubspecies rice hybrids. In general, most of these hybrids mature late, which thus limits their cultivated adaptability. We believe that the deployment of the *Ef-cd* locus which makes maturity occur earlier without yield penalty can improve these hybrids to be adapted to new ecological areas and production seasons, leading to more extensive cultivation.

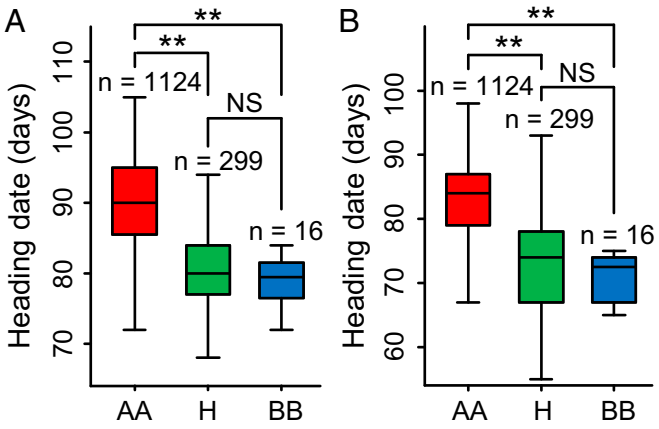

Fig. 4. Analysis of genotype and phenotype interaction of *Ef-cd* among elite hybrid cultivars. Hybrid rice varieties with early maturing NIL D248 homozygous (BB) and heterozygous (H) genotype headed significantly earlier than those with its wild type SH881 genotype (AA) in Sanya (*A*) and Hangzhou (*B*). The *P* value was calculated using Student's *t* test. $^{**}P < 0.01$; NS, not significant.

Materials and Methods

During all stages of developing early maturing NILs using 6 late-maturing recurrent parents and the early maturing donor line 6442S-7 (*SI Appendix*, Fig. S1*A*), the genotypes of offspring plants were analyzed for the *Ef-cd* locus using markers RM231, RM22, C515, and InDel1 (14) (Dataset S4), and the homozygotes were selected for continuous backcrosses and self-pollinations until the NILs of *Ef-cd* became genetically stable. Two recurrent parents of SH881 and MH63 and their 2 early maturing NILs D248 and D330 were selected for phenotyping the major agronomic traits of Beijing in 2009. Details on the following are available in *SI Appendix, SI Materials and Methods*: material preparation and growth conditions, fine mapping of *Ef-cd*, vector construction and plant transformation, RNA isolation and quantitative RT-PCR, RACE, LUC assays, labeling with ^{15}N-nitrate or ^{15}N-ammonium for determination of ^{15}N accumulation, photosynthesis-related parameter measurements, ChIP assay, strand-specific RNA sequencing and ChIP-seq data analysis, analysis of differentially expressed genes, and accession numbers. The primers used in this study are listed in Dataset S4.

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