Fine-tuning flowering time via genome editing of upstream open reading frames of Heading date 2 in rice

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Abstract

Flowering time of rice (*Oryza sativa L.*) is among the most important agronomic traits for region adaptation and grain yield.In the process of rice breeding, efficient and slightly modulating the flowering time of an elite cultivar would be more popular with breeder. Hence, we focus on slightly increasing the expression of flowering repressors by CRISPR/Cas9 genome editing system. It was predicated there were three uORFs in 5' leader sequence of *Hd2*.By editing Hd2 uORFs, we got four homozygous mutant lines and phenotypic analysis showed their heading date delay by 4.6–11.2 days relative to wide type SJ2.The mutation do not affect transcript level of *Hd2*, but improve translation of a downstream Hd2 pORF. In summary, we developed a efficient approach for delaying rice heading date based on editing uORF region of flowering repressor, which is time and labor saving compared to traditional breeding. In future, uORF of other flowering time related genes, including flowering promoter and flowering repressor genes, can also be used as targets to fine-tune the flowering time of varieties.

Main Text

Rice, as one of the most important cereal crops, is staple food for more than half of the human population. Rice growing region span a large latitude (from 40°S to 53°N). To adapt to the local circumstance, rice need to flower and ripe at the right time. Flowering time (also called heading date in rice) is thus one of the most important agronomic traits, and determines rice distribution and final yield. Flowering time is a very complicated trait which is controlled by multiple internal genetic factors and diverse external environmental factors (Hori et al. 2016; Cho et al. 2017). Over the past few decades, extensive molecular genetics studies have identified numerous genes involved heading date in rice (Hori et al. 2016). It was shown that various combination of diverse heading date related genes with multiple natural variations finally determines the local adaptability of a specific cultivar (Gao et al. 2014; Li et al. 2015; Zhang et al. 2015; Fujino et al. 2019).

Rice is a short-day (SD) plant and flowering time of rice is very sensitive to day length (Hori et al. 2016). SD condition promotes flowering and long day (LD) condition delays flowering (Hori et al. 2016). Therefore, photoperiod pathway is the most important flowering time regulatory pathway in rice (Song et al. 2013). Rice has two major photoperiod-dependent flowering pathways, an evolutionarily conserved OsGI-Hd1-Hd3a pathway and an specific Ehd1-Hd3a pathway(Hori et al. 2016). Ehd1 activates the expression of two florigens Heading date 3a (Hd3a) and RICE FLOWERING LOCUS T1 (RFT1) to promote flowering (Doi et al. 2004).

Both Hd1 and Ehd1 are regulated by diverse flowering time regulators via modulating the transcription or protein level of Hd1 and Ehd1 (Hori et al. 2016). In Hd1-dependent pathway, Hd1 is an activator under SD condition, while acts as a suppressor under LD (Yano et al. 2000). Hd1 protein can be phosphorylated by OsK4 and ubiquitinated by HAF1 at post-translation level(Yang et al. 2015; Sun et al. 2016). In Ehd1-dependent pathway, Ehd1 is regulated by multiple suppressors (e.g. Hd2, Hd4, Hd5, OsCOL4, OsCOL10, OsLFL1) and activators (e.g. DTH3, OsMADS51, OsMADS56, Ehd2, Ehd4) (Hori et al. 2016).

In the process of rice breeding, efficient and slightly modulating the flowering time of an elite cultivar would be more popular with breeder. CRISPR/Cas9 genome editing system have been proved a favorable technique for breeding, and a number of major traits so far have been edited successfully (Mao et al. 2019). Previous studies have shortened the rice flowering time through editing the diverse flowering repressor genes (Li et al. 2017; Cui et al. 2019). However, it is still needed to delay the flowering time in rice breeding. To meet this demand, there were two options, increasing the expression of flowering repressor or decreasing the expression of the flowering promoter. In rice flowering regulatory pathway, there were less flowering promoter genes than flowering repressor genes (Hori et al. 2016). In addition, the effect of flowering promoter genes (e.g. Ehd1, Hd3a, and RFT1) is too strong to be used as target genes of editing, since the loss of function of Ehd1, Hd3a, and RFT1 will strikingly delay flowering or never flower, which limit their practical breeding values(Zhao et al. 2015).

Hence, we plan to slightly increase the expression of flowering repressor, and generate continuous subtle delay-flowering phenotypes. It was reported that upstream open reading frames (uORFs) can repress the translation of downstream primary ORFs (pORFs), and the protein level of pORFs can be increased via editing and mutating the uORFs (Zhang et al. 2018). Followed this logic, we sought to search and mutate the uORFs in the flowering repressor genes via genome editing. Through online searching in plant uORFs database (http://uorflight.whu.edu.cn/)(Niu et al. 2020), it was predicated there were three uORFs in 5' leader sequence of *Hd2*. These uORFs were named as uORF 1, uORF 2 and uORF 3, and chosen as targets for genome editing (Fig. 1A).

First, we designed three sgRNAs to target uORF 1, uORF 2 and uORF 3 respectively (Fig. 1B). These sgRNA catastases were sequentially ligated into the CRISPR/Cas9 binary vectors p*YLCRISPR/Cas9P*$_{ubi}$*-H* (Ma et al. 2015). Songjing 2, an elite rice variety grown in Heilongjiang province, was chosen for transformation. At T_0 generation, we got 12 independent transgenic lines (Fig. S1). In the T_1 generation, mutation types of uORF 1, uORF 2 and uORF 3 in these lines were determined through sequencing (Table S1). Four homozygous mutant seedlings with different mutation types, *uorf hd2-1* to *uorf hd2-4* , were identified and used for further investigation. (Fig. 1C). We found that uORF 1 was edited in all four lines, uORF 2 was edited in two lines, and uORF 3 was not edited in four lines (Fig. 1C). In addition, the mutation sites for uORF 1 target are very close to ATG, and initiation codon of uORF1 was deleted in *uorf hd2-3* and *uorf hd2-4* (Fig. 1C). These results suggested that uORF 1 is a more efficient target.

At T_2 generation, four homozygous lines *uorf hd2-1* to *uorf hd2-4* were investigated. Phenotypic analysis showed that *uorf hd2-1* to *uorf hd2-4* delay flowering by 4.6-11.2 days, respectively, relative to wide type SJ2, and *uorf hd2-3* and *uorf hd2-4* delay flowering more severe than *uorf hd2-1* and *uorf hd2-2* (Fig. 1D, E). Supporting this result, qRT-PCR analysis showed the expression of florigen gene *Hd3a* and *RFT* is significant decreased in *uorf hd2-1* to *uorf hd2-4* compared with SJ2 (Fig. 1F, G). In addition, we also examined the expression of *Hd2*, and its transcript level is comparable between SJ2 and four edited lines (Fig. S2). This result indicates the mutated uORFs of *Hd2* do not affect the transcription of pORF of *Hd2*, which is consistent with previous report (Zhang et al. 2018). It has been shown that uORF performs its function *via* suppressing the translation efficiency and protein level of pORF (Zhang et al. 2018). We thus

attempted to examine the protein level of Hd2 in *uorf hd2-1* to *uorf hd2-4*. However, we did not get effective Hd2 antibody. To overcome this obstacle, we tested whether mutated Hd2 uORF affect translation of a downstream Hd2 pORF in rice protoplast system combined with the dual luciferase reporter system. As shown in Fig. 1H, five constructs were made in which wild type *Hd2* uORF and four mutated hd2 uorf were inserted between the *35S* promoter and LUC reporter. The translation level of LUC was recorded by reading the ratio of LUC and REN. We found that the expression of LUC directed by *35S* promoter is very high, while the insertion of Hd2 uORF largely suppress the expression of LUC (Fig. 1I). However, when the Hd2 uORF is replaced by hd2 uorf, the expression of LUC is significantly recovered to different extent, indicating that the Hd2 uORF indeed suppress the expression Hd2 pORF (Fig. 1I). Additionally, the recovered amount in hd2 uorf 3 and hd2 uorf 4 is much more than that in hd2 uorf 1 and hd2 uorf 2 (Fig. 1I). These results agreed with the diversity of delaying flowering phenotype in *uorf hd2-1* to *uorf hd2-4* (Fig. 1E).

In summary, we developed a efficient approach for delaying rice heading date based on editing uORF region of flowering repressor, which is time and labor saving compared to traditional breeding. In future, uORF of other flowering time related genes, including flowering promoter and flowering repressor genes, can also be used as targets to fine-tune the flowering time of varieties.

Abbreviations

SD:short-day;LD:long day;GI:GIGANTEA;Hd1:Heading date1;Ehd1:Early heading date1;Hd3a:Heading date 3a;RFT1:RICE FLOWERING LOCUS T1;HAF1:Heading date Associated Factor 1;Hd2:Heading date2;Hd4:Heading date4;Hd5:Heading date5;COL4:CONSTANS-like 4;COL10:CONSTANS-like 10;OsLFL1:LEC2 and FUSCA3 like 1;DTH3:Days to Heading 3;Ehd2:Early Heading Date 2;Ehd4:Early Heading Date 4;CRISPR:Clustered regulatory interspaced short palindromic repeat;uORFs:upstream Open Reading Frames;pORF:primary Open Reading Frame;LUC:Firefly luciferase;REN:Renilla luciferase

Declarations

Ethical Approval and Consent to participate

Not applicable

Consent for publication

Not applicable

Availability of supporting data

All data generated or analyzed during this study are included in this published article and its supplementary information files.

Competing interests

The author(s) declare that they have no conflict of interest.

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Authors' contributions

QYB and ZYW designed and supervised the research. XXL, YYZ, HLL and MLH performed the experiments. WM and RTL analyzed data. XFL wrote the paper. The author(s) read and approved the final manuscript.

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Figures

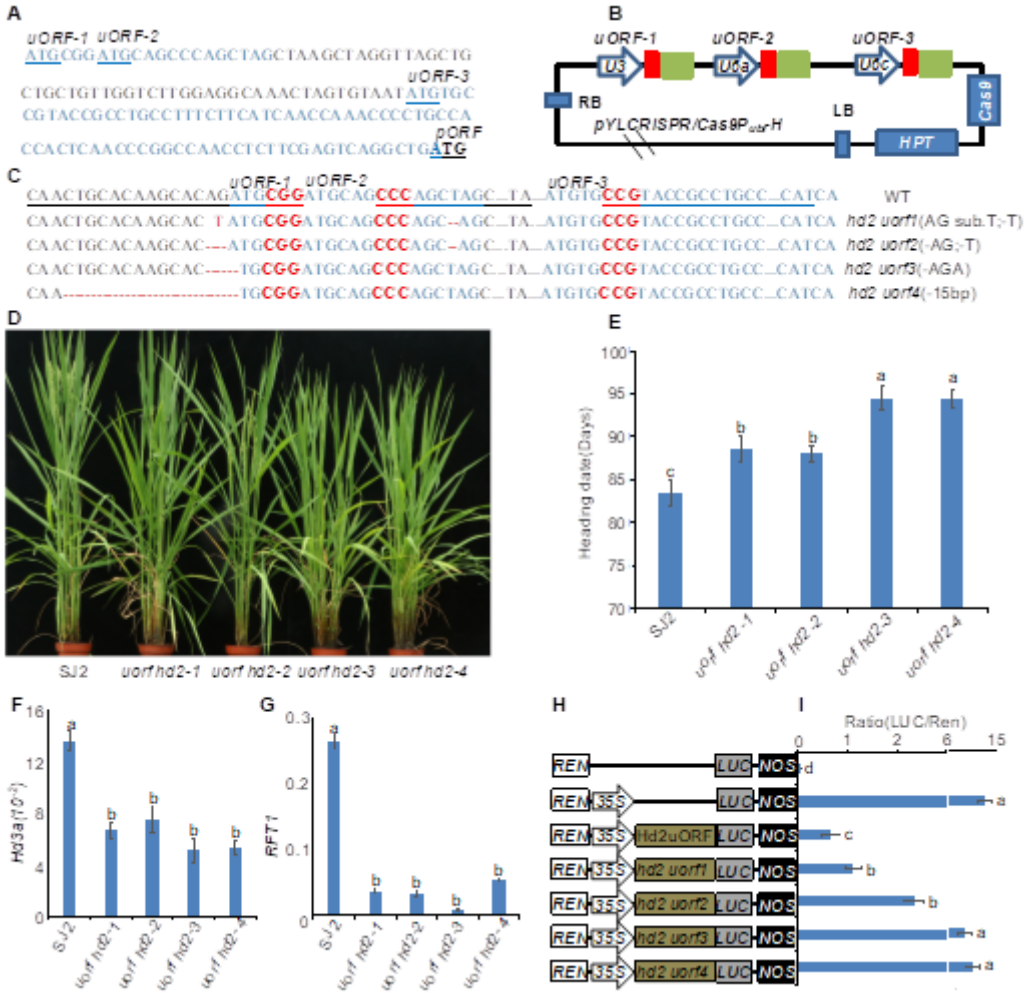

Figure 1

Editing of Hd2 uORFs delays rice heading date. (A) There are three uORFs in 5' leader sequence(blue) of Hd2, and uORFs putative initiation codon is underlined.pORF is shown in bold with underlined. (B) Schematic diagram of the CRISPR/Cas9 vector. Three target sequences (uORF-1,uORF-2,uORF-3) fused with sgRNA scaffolds were driven by respective U3 or U6 promoters. The three sgRNA expression cassettes were sequentially inserted into the binary vector pYLCRISPR/Cas9Pubi-H. Red boxes indicate three target sequences and green boxes indicate sgRNA scaffolds. HPT, hygromycin phosphotransferase gene. (C) Four homozygous mutants (T2 generation) of hd2 uorf1 to hd2 uorf4 obtained by CRISPR/Cas9 editing. The uORF sequence (blue) is shown with the sgRNA target site underlined and the protospacer-adjacent motif shown in red. The nucleotide changes are labeled in red. '-' means deletion and sub. means substitution. (D) Representative flowering image of mutants uorf hd2-1 to uorf hd2-4 indicated genotypes under NLD in summer at Harbin. Wide type SJ2 was used as control. (E) Flowering time of each genotype under NLD conditions. Data are means±SE (n=20). (F) and (G) qRT-PCR analysis of Hd3a (F) and RFT1 (G) transcription level in indicated lines and SJ2. Rice UBIQUITIN gene was used as the internal control. Means and standard deviations were obtained from three biological replicates. Data are means±SE (n=3). (H) Schematic diagrams of the reporter plasmids used in rice protoplasts transient assay. REN, Renilla luciferase; LUC, firefly luciferase. (I) The LUC activity in rice protoplasts with indicated

reporter plasmids. Data are means±SE (n=3). Statistically significant differences are indicated by different lowercase letters ($P < 0.05$, one-way ANOVA with Tukey's significant difference test).

Supplementary Files

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