***Heading date 1 (Hd1)*, an ortholog of *Arabidopsis* *CONSTANS*, is a possible target of human selection during domestication to diversify flowering times of cultivated rice**

Yasuyuki Takahashi[†] and Ko Shimamoto^{*}

Laboratory of Plant Molecular Genetics, Nara Institute of Science and Technology,
8916-5 Takayama, Ikoma, Nara 630-0101, Japan

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During the domestication of rice (*Oryza sativa* L.), diversification of flowering time was important in expanding the areas of cultivation. Rice is a facultative short day (SD) plant and requires certain periods of dark to induce flowering. *Heading date 1 (Hd1)*, a regulator of the florigen gene *Hd3a*, is one of the main factors used to generate diversity in flowering. Loss-of-function alleles of *Hd1* are common in cultivated rice and cause the diversity of flowering time. However, it is unclear how these functional nucleotide polymorphisms of *Hd1* accumulated in the course of evolution. Nucleotide polymorphisms within *Hd1* and *Hd3a* were analyzed in 38 accessions of ancestral wild rice *Oryza rufipogon* and compared with those of cultivated rice. In contrast to cultivated rice, no nucleotide changes affecting *Hd1* function were found in 38 accessions of wild rice ancestors. No functional changes were found in *Hd3a* in either cultivated or ancestral rice. A phylogenetic analysis indicated that evolution of the *Hd1* alleles may have occurred independently in cultivars descended from various accessions of ancestral rice. The non-functional *Hd1* alleles found in cultivated rice may be selected during domestication, because they were not found or very rare in wild ancestral rice. In contrast with *Hd3a*, which has been highly conserved, *Hd1* may have undergone human selection to diversify the flowering times of rice during domestication or the early stage of the cultivation period.

Key words: flowering time, domestication, cereal, natural variation, polymorphism

INTRODUCTION

Rice is one of the most important cereal crops in the world. The domestication and breeding of rice has occurred during the last 8000 years (Khush, 1997; Doebley et al., 2006). Cultivated rice was originally a tropical plant that evolved from a wild rice species, *Oryza rufipogon* (Garrius et al., 2005; Caicedo et al., 2007). For the origin of cultivated rice, single or multiple domestication events have been proposed. Archaeological studies on ancient rice grains support single domestication event. In former hypothesis, *O. sativa indica* varieties were considered to be domesticated from *O. rufipogon*. Then, *O. sativa japonica* varieties were developed from

indica rice (Oka and Morishima, 1982; Chen, 1999). On the other hand, the latter hypothesis which was supported by several genetic distance studies argues that *japonica* and *indica* varieties were independently domesticated from different ancestral wild rice species (Londo et al., 2006; Sweeney and McCouch, 2007b). *O. rufipogon* grows in tropical and sub-tropical regions of Asia and Oceania. During rice domestication, *O. sativa* acquired various agriculturally important morphological and physiological traits. These include reductions in grain shattering, yield increases, and changes in plant architecture, seed dormancy, starch quality, and seed size (Kovach et al., 2007; Izawa et al., 2009). Genetic studies on these domestication traits have been performed in rice. For example, *qSH1* and *sh4* were found by QTL mapping and related to grain shattering which is one of the well studied domestication traits (Li et al., 2006; Konishi et al., 2006; Lin et al., 2007; Zhang et al., 2009). *Rc* encodes a bHLH protein which causes the change from red grain to white grain (Sweeney et al., 2006, 2007a). Furthermore, grain

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^{*} Corresponding author. E-mail: simamoto@bs.naist.jp

[†] Present address: Max Planck Institute for Plant Breeding Research, Carl von Linne Weg 10, D-50829 Cologne, Germany

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stricture is also an important domestication trait. GW2, qSW5, and GS3 were known as domestication related genes which improved the grain shape (Song et al., 2007; Shomura et al., 2008; Takano-Kai et al., 2009).

Flowering time was also altered during the domestication process. Diversification of flowering times enabled farmers to expand the growing area (Izawa, 2007; Jung and Muller, 2009). Induction of rice flowering is mainly regulated by day length, and flowering is induced under SD conditions. The genetic pathways that control photoperiodic flowering in rice have been well studied, and a number of genes involved in this regulation are known (Tsuji et al., 2008, 2011; Komiyama et al., 2009). *OsGI*, an ortholog of *Arabidopsis GIGANTEA*, is under the control of the circadian clock and regulates *Hd1* expression (Hayama et al., 2002, 2003). *Hd1*, an ortholog in *Arabidopsis CONSTANS*, regulates expression of the *Hd3a* florigen gene (Yano et al., 2000; Hayama et al., 2002; Izawa et al., 2002). *Hd1* is a zinc finger type transcriptional activator carrying the conserved CCT (*CO*, *CO-like*, *TOC1*) domain. The CCT domain functions as a nuclear localization signal and the lack of this domain causes a defect in protein function (Robson et al., 2001). *Early heading date 1 (Ehd1)* encodes a B-type response regulator that acts independently of *Hd1* as a floral regulator, and its ortholog has not been found in *Arabidopsis* (Doi et al., 2004). Therefore, *Ehd1* is a rice-specific flowering regulator. *Hd1* and *Ehd1* independently regulate *Hd3a* expression under SD conditions (Tsuji et al., 2008; Komiyama et al., 2009). *Hd3a* is a rice ortholog of *Arabidopsis FLOWERING LOCUS T (FT)* and encodes a florigen, which is a mobile flowering signal (Kojima et al., 2002; Tamaki et al., 2007; Corbesier et al., 2007; Jaeger and Wigge, 2007; Mathieu et al., 2007). Furthermore, *RICE FLOWERING LOCUS T1 (RFT1)*, which belongs to the *FT*-like gene family, encodes a second floral activator that mainly works under long day (LD) conditions (Komiyama et al., 2008, 2009).

We have previously reported on the molecular mechanisms that promote diversity in flowering times in cultivated rice, and showed that *Hd1* is one of the major determinants of flowering time (Takahashi et al., 2009). Five different types of nucleotide changes that critically affect the protein activity of *Hd1* were found. Nine of the

17 *Hd1* alleles analyzed encoded non-functional *Hd1* proteins. Furthermore, cultivars with non-functional *Hd1* alleles showed significantly later flowering times than those with functional *Hd1* alleles (Takahashi et al., 2009). These results suggest that functionally important nucleotide polymorphisms in the *Hd1* coding region have contributed to the diversity in flowering times in cultivated rice. However, it is not known whether these nucleotide polymorphisms arose before or during domestication. To gain some insight into when these variations in *Hd1* occurred, we analyzed the nucleotide polymorphisms of *Hd1* in the progenitor of cultivated rice, *O. rufipogon*.

MATERIALS AND METHODS

Plant materials and growth conditions The *O. rufipogon* accessions were retrieved from the Oryzabase (<http://www.shigen.nig.ac.jp/rice/oryzabase/top/top.jsp>) and supplied by the National Institute of Genetics (Supplementary Table S1). The *O. sativa* core collection was obtained from the National Institute of Agrobiological Sciences Genebank (Kojima et al., 2005; Supplementary Table S2). *O. sativa Hd1* and *Hd3a* alleles used in this study are shown in Takahashi et al. (2009). The plants were grown in climate chambers under short day conditions with daily cycles of 10 h light at 30°C and 14 h dark at 25°C. Fluorescent white light (400 to 700 nm, 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$) was provided at 70% humidity.

Sequence analysis The coding regions of each gene were amplified using PrimeSTAR HS DNA polymerase (Takara) from genomic DNA, and the PCR products were purified using the MinElute system (QIAGEN). These purified PCR fragments were used as templates, and sequencing reactions were performed using the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Data were collected using the ABI PRISM 3100 Genetic Analyzer and analyzed using the GENETYX program ver.7 (GENETYX, Tokyo, Japan).

RNA extraction and cDNA synthesis Leaves were harvested from plants grown under SD conditions 35 days after germination, at the zeitgeber time 16, which corresponds to the expression peak of *Hd1*. Leaf tissues were

Table 1. Summary of polymorphisms in flowering time genes in rice

Gene (Acc. no. ^a)	Species	No. of acc.	SNPs ^b		Indel sites	F. S. sites ^c	Nonsense mutation	No. of alleles
			Synonymous	Nonsynonymous				
<i>Hd1</i>	<i>O. sativa</i>	60	1	13	8	4	1	17
(AB041840)	<i>O. rufipogon</i>	38	31	66	17	0	1	33
<i>Hd3a</i>	<i>O. sativa</i>	64	3	4	0	0	0	6
(AB052944)	<i>O. rufipogon</i>	38	8	12	0	0	0	20

^a GenBank accession number. ^b Coding sequence without indel region. ^c F. S., frame shift.

ground in liquid nitrogen with the ShakeMaster Auto ver. 2.0 (BioMedicalScience Inc.). Total RNA was extracted using TRIzol (Invitrogen) and treated with DNase I (Invitrogen). cDNA (20 μ l) was synthesized from 1 μ g of total RNA using SuperScriptII Reverse Transcriptase (Invitrogen).

Constructs for the luciferase reporter assay The *Hd1* coding region of each *O. rufipogon* accession was amplified by PCR from cDNA using PrimeSTAR HS DNA polymerase (Takara). *Hd1* fragments were subcloned into the pENTR/D-TOPO vector using the Directional TOPO Cloning kit (Invitrogen). Subsequently, the *Hd1* was transferred to the CaMV 35S::Gateway cassette plasmid, which contains a pUC12 backbone, by performing an LR recombination reaction (Invitrogen). The *Hd3a* promoter sequence was cloned from cv. Nipponbare. This fragment was subcloned into pUC12 along with the luciferase coding region (*Photinus pyralis*) using the Ligation-Convenience Kit (NIPPON GENE).

Luciferase reporter assays Protoplasts (2×10^5 cells) were prepared from rice Oc cell cultures and transformed using the PEG transfection method (Chen et al., 2006). The cells were transformed with: 1 μ g of the *pHd3a::pLUC* construct as a reporter, and 5 μ g of each *Hd1* construct containing the *Hd1* coding sequence driven by the CaMV 35S promoter. For a vector control, the bialaphos-resistance gene (*Bar*) was used instead of the *Hd1* coding sequence. After incubation for 12 h, luciferase activities were measured using a Luciferase Assay System (Promega). The luminescence derived from each *Hd1* allele was normalized for sample protein level and evaluated as a proportion of that of the vector control. Statistical analyses were performed using Microsoft Excel 2003 or Statcel 2 (OMS, Tokyo, Japan).

Phylogenetic analysis The alignment was done using the program GENETYX program ver.7 (GENETYX, Tokyo, Japan) and afterwards inspected carefully and adjusted manually. Phylogenetic reconstruction was done by Neighbor-Joining method (Saitou and Nei, 1987) using the program MEGA5 (Tamura et al., 2011). Bootstrap values were calculated using the same program, number of replicates set to 10,000 (Felsenstein, 1985). The evolutionary distances were calculated using the Maximum Composite Likelihood method (Tamura et al., 2004) and are in the units of the number of base substitutions per site. Gaps were coded as A/T matrix at the end of the alignment.

RESULTS

Nucleotide polymorphisms in the *O. rufipogon* *Hd1* coding region We obtained all 38 *O. rufipogon* accessions from 277 accession of wild rice core collection (The

Fig. 1. Comparison of nucleotide polymorphisms within the *Hd1* coding sequences of *O. sativa* and *O. rufipogon*. Sites that are polymorphic in nucleotide and amino acid sequences are shown. Red arrowheads indicate the sites of polymorphisms shared by both *O. sativa* and *O. rufipogon*. The locations of nucleotide changes that cause loss of protein function are boxed.

Fig. 2. Activities of *Hd1* alleles determined using transient luciferase reporter assays in rice protoplasts. The *Hd1* alleles of cv. Nipponbare and cv. Kasalath were used as functional and nonfunctional controls, respectively. The bialaphos resistance gene (*Bar*) was used as a vector control. Error bars represent S.D., *n* = 6.

National BioResource Project, Japan), which were derived from all over the world and represent the diversity of wild rice accessions in 18 species from 9 genomes covering AA, BB, CC, BBCC, CCDD, EE, FF, GG and HHJJ. Our sequence analysis identified 33 *Hd1* alleles with 97 SNPs among them (Table 1). Among the SNPs, 31 were synonymous and 66 were non-synonymous nucleotide changes. Furthermore, 12 deletions, 5 insertions, and one nonsense mutation were identified, but all the deletions and insertions were in-frame and thus no frame-shift mutations were detected (Fig. 1). The one nonsense mutation (position 1217) is at the very end of the coding sequence, and this mutation does not seem to affect protein function. Comparison with the *O. sativa* *Hd1* alleles revealed 10 identical SNPs and 3 common alleles; the *O. rufipogon* W120, W1852, and W1715 alleles are identical to the *O. sativa* Type 1, Type 4 and Type 5 *Hd1* alleles, respectively. Our previous study identified 5 different nucleotide changes in the *Hd1* coding region of *O. sativa*: four frame-shift and one nonsense mutation, all of which cause defects in the CCT domain (Takahashi et al., 2009). Interestingly, these nucleotide changes were not found in *O. rufipogon*, suggesting that the non-functional *Hd1* mutations found in cultivated rice may have arisen during domestication.

Functional assays of *O. rufipogon* *Hd1* alleles
Although no frame-shift or nonsense mutations were detected in the *Hd1* gene of *O. rufipogon*, several amino acid substitutions were found in the two conserved regions, the zinc finger and the CCT domains (Fig. 1). Furthermore, 11 other amino acid substitutions were found in other conserved regions. Because it was unclear whether these altered Hd1 proteins are func-

Fig. 3. Comparison of nucleotide polymorphisms within the *Hd3a* coding sequences of *O. sativa* and *O. rufipogon*. Sites that are polymorphic in nucleotide and amino acid sequences are shown. Red arrowheads indicate the sites of polymorphisms shared by both *O. sativa* and *O. rufipogon*.

tional, we performed transient functional assays in rice protoplasts (Hayama et al., 2002; Takahashi et al., 2009). In this assay, the overexpression of a functional *Hd1* gene suppresses *Hd3a* promoter activity, which is evaluated using the luciferase reporter (Hayama et al., 2002; Takahashi et al., 2009). We examined 11 *Hd1* alleles *O. rufipogon* and found that all the alleles examined were functional (Fig. 2). This result clearly demonstrated that *Hd1* is highly conserved and functional in *O. rufipogon*.

Nucleotide polymorphisms in the *O. rufipogon* *Hd3a* coding region Under SD conditions, *Hd1* regulates expression of the florigen encoding gene (Hayama et al., 2003; Tamaki et al., 2007; Komiya et al., 2009). Therefore, we also investigated the nucleotide polymorphisms in the *Hd3a* coding region of *O. rufipogon*. The sequence analysis revealed 20 *Hd3a* alleles (encoding 17 different amino acid sequences) and 20 SNPs among the 38 accessions. Eight SNPs were synonymous, and 12 were non-synonymous nucleotide changes, and no indels were identified (Fig. 3, Table 1). Three alleles are shared between *O. sativa* and *O. rufipogon*: the *O. rufipogon* W630 allele is identical to the *O. sativa* Type 2 allele; the *O. rufipogon* W1921, W2263, and W2265 alleles

are identical to *O. sativa* Type 3; and *O. rufipogon* W1852 is identical to *O. sativa* Type 6. Mutation sites that have previously been identified in *Arabidopsis FT* were not altered in the *Hd3a* gene of *O. rufipogon* (Pnueli et al., 1998, 2001; Kobayashi et al., 1999; Hanzawa et al., 2005). These results indicate that *Hd3a* function is highly conserved in both of the *Oryza* species. Furthermore, a comparison of *Hd3a* sequences in the two species indicated that almost all the nucleotide changes found in *O. sativa* are conserved in *O. rufipogon*, suggesting that very few nucleotide substitutions occurred during domestication. This situation is in strong contrast to that of *Hd1* (Fig. 1).

Phylogenetic analysis of *Hd1* alleles in *O. sativa* and *O. rufipogon* To understand how various *Hd1* alleles of *O. sativa* evolved from those in ancestral wild rice, we generated a phylogenetic tree of all the alleles. Several well-separated clusters were identified, and some of these contained alleles from both *O. sativa* and *O. rufipogon*. This suggested that the *Hd1* alleles of cultivated rice may have evolved independently from multiple *O. rufipogon* accessions. Indeed, three alleles were found in both *O. sativa* and *O. rufipogon*, and the *O. rufipogon* accessions containing these alleles were col-

Fig. 4. A phylogenetic tree of *Hd1* alleles from *O. sativa* and *O. rufipogon*. The ecotypes of *O. rufipogon* strains were shown in A, annual; P, perennial. The *Hd1* alleles of *O. sativa* are classified as Types 1–17, and the number of each subspecies (In., Indica; Ja., Japonica) with each allele type is given. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (10,000 replicates) is shown next to the branches. The scale bar of genetic distance is shown at the bottom.

lected in India (W120), Thailand (W1852), and China (W1715) (Fig. 4, Supplementary Fig. S1). Furthermore, several clusters contained both functional and non-functional *Hd1* alleles from *O. sativa*. These results suggest that the non-functional *Hd1* alleles of cultivated rice may have arisen during domestication or the early stage of the cultivation period, and that *Hd1* may be one of the targets of selection during rice breeding.

DISCUSSION

Although the *Hd1* alleles of *O. sativa* are generally less polymorphic than those of *O. rufipogon*, they are functionally more diverse due to the presence of functionally important mutations (Takahashi et al., 2009). Five different types of functionally important mutations have been found in the *O. sativa* *Hd1* gene (Takahashi et al., 2009). Since ca. 8000 years of rice domestication is very short to accumulate these mutations in *O. sativa* it is likely that *Hd1* has gone through strong positive selection to diversify its function. The very rapid evolution of genes during domestication may be generally caused by high mutation frequency and strong selective advantages of mutations. In the case of rice *Hd1*, mutations having selective advantage are frame-shift and nonsense mutations. Since these mutations appear with much higher frequencies than those with selective advantages this may have caused the unique evolution of rice *Hd1* during domestication. We also note that the most of *O. sativa* cultivars used in this study are landrace, and no specific mutations of *Hd1* were observed in modern cultivars (Supplementary Table S2). Therefore, almost all the mutations of *Hd1* may not be selected in modern breeding during 20th century but during domestication including the early stage of the cultivation period.

Domestication-related genes have been reported as factors associated with agronomically important traits (Kovach et al., 2007; Izawa et al., 2009). Flowering time genes are also related to domestication since their expression has been modified to expand cultivation areas and to alter various breeding styles (Izawa, 2007; Izawa et al., 2009; Jung and Muller, 2009). For example, the growth of rice plants is strongly affected by temperature; low temperatures during pollen formation cause decreases in pollen fertility. Therefore, to expand rice cultivation to regions of high latitude, the timing of flowering and fertilization must be altered so that they occur before the arrival of the cold season. However, higher latitude regions have longer day lengths in the summer, and this is unfavorable for rice flowering. *Hd1* activates flowering under SD conditions, but under LD conditions *Hd1* represses flowering (Hayama et al., 2003; Komiya et al., 2009). Therefore, mutations that suppress *Hd1* activity make rice less sensitive to SD conditions, and result in rice cultivars that can be grown at higher latitudes. In

this sense, *Hd1* can be defined as a “diversification” gene rather than a domestication gene (Gross and Olsen, 2010).

We found that functionally important mutations of *Hd1* emerged independently in various regions of Asia. The fact that *O. sativa* and *O. rufipogon* share several *Hd1* alleles suggests that various domestic rice cultivars inherited *Hd1* independently from different accessions of ancestral wild rice. This is consistent with a model of cultivated rice arising through multiple domestications (Kovach et al., 2009). The decreased *Hd1* activity may have played a role in expanding the growing area to high latitude regions during the early stages of domestication. On the other hand, there is some overlap in the growth areas of cultivated rice and *O. rufipogon* in tropical areas (Takahashi et al., 2009). This could be explained by the fact that changes in flowering time contribute not only to the expansion of cultivation areas but also to yield. Delayed flowering caused by a decrease in *Hd1* activity may result in higher yields. This is also supported by the entangled evolution of the *Hd1* alleles (Fig. 4). Crossing with other rice strains and human selection may have also accelerated the evolution of diversity in flowering time.

We found that most of the cultivars which possess the same non-functional *Hd1* alleles are widely distributed in Asian countries (Supplementary Table S2), indicating that the acquisition of non-functional *Hd1* alleles might have occurred before expansion of the cultivation areas. On the other hand, *Hd1* allele of W120 (Type1 *Hd1* allele) is identical to Type 2, 3 and 7 non-functional *Hd1* alleles except for the frame-shift mutation. Although W120 was collected from India, cultivars carrying Type 2, 3 and 7 non-functional *Hd1* alleles are derived from Philippines, China, Korea and Myanmar. Therefore, at this moment it is difficult to determine whether these non-functional *Hd1* alleles have directly arisen from ancestral wild strains or from the rice cultivars with Type 1 *Hd1* alleles. To answer this question, further study using more detailed geographical information need to be performed.

We notice that the data in this study do not preclude the possibility that some of the non-functional *Hd1* alleles exist in other *O. rufipogon* accessions that were not examined in this study. Furthermore, in the past, some *O. rufipogon* populations could contain non-functional *Hd1* alleles under certain ecological conditions which are different from now. At this stage of research, therefore, our conclusions should be treated as a hypothesis that requires further testing with larger scale of wild rice materials and investigation for nucleotide diversity in surrounding genomic region to examine the evidence for a selective sweep. Furthermore, the frequency of many deleterious mutations which was accumulated in the absence of effective recombination during domestication artificial selection has been reported (Lu et al., 2006; Cruz

et al., 2008). It is clear that defective mutations in *Hd1* generate the diversity of flowering time in *O. sativa* cultivars (Takahashi et al., 2009), but the detailed investigation should be required to firmly conclude the Hd1 is a target of human selection during rice domestication.

In this study we focused on the molecular evolution of *Hd1* during rice domestication. In the future it will be interesting to investigate how other flowering regulators function in *O. rufipogon*. *Ehd1* is an important regulator of *Hd3* expression (Doi et al., 2004), and variations in *Ehd1* expression were also shown to contribute to the diversity of flowering time in cultivated rice (Takahashi et al., 2009). *Ghd7*, which is a negative regulator of *Ehd1* and flowering, is another attractive candidate for further investigation (Xue et al., 2008). Furthermore, *RFT1*, the closest homolog to *Hd3a*, is a floral regulator under LD conditions (Komiya et al., 2008, 2009). We also showed that *cis*-regulatory region of *Hd3a* is a potential factor generating diversity in flowering time (Takahashi et al., 2009). The analysis of all these flowering time genes will contribute greatly to our understanding of the variations in flowering induction during rice domestication.

CONCLUSIONS

In this study we examined two key regulators of flowering time in rice: *Hd1* and *Hd3a*. We analyzed their evolution during domestication by comparing their nucleotide polymorphisms in *O. sativa* and *O. rufipogon*. Our analysis showed that in contrast with cultivated rice, no functional variations in *Hd1* were found in 38 accession of wild ancestral rice. Furthermore, a phylogenetic analysis showed that the *Hd1* alleles in cultivated rice might have evolved at multiple independent locations. Together, these results suggest that the non-functional *Hd1* alleles found in cultivated rice originated during domestication. In contrast to *Hd1*, very few nucleotide polymorphisms that affect protein function were found in the *Hd3a* florigen-encoding gene of either *O. sativa* or *O. rufipogon*. Decreases in Hd1 activity may have been desirable for the expansion of rice cultivation to higher latitude areas, and in the development of varieties adapted to various environments at multiple locations around the world. Thus, *Hd1* may have been a target of human selection to diversify flowering time during rice domestication or the early stage of the cultivation period.

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