African Journal of Biotechnology Vol. 9(23), pp. 3368-3372, 7 June, 2010
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB10.297
ISSN 1684–5315 © 2010 Academic Journals

Full Length Research Paper

Molecular marker analysis of heading date *Hd1* locus in Egyptian rice varieties

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Accepted 10 May, 2010

Nine molecular markers derived from the heading date QTL *Hd1* DNA sequence for cultivated rice were used to study the heading date allelic diversity of the cultivated Egyptian rice varieties. The results showed that among the nine simple sequence repeats (SSR) and sequence tagged-sites (STS) markers used, one SSR marker, Hd1AGC, amplified three polymerase chain reaction (PCR) fragments (alleles) of various sizes (441, 490, and 620 bp) in all rice varieties, while the rest of the markers amplified only one non-polymorphic PCR fragment. The study also exhibited that Hd1AGC amplified a unique PCR fragment (620 bp) linked to early heading date in Giza 177 and Sakha 103.

Key words: Flowering time, simple sequence repeats, *Oryza sativa* L., heading date, sequence tagged-sites.

INTRODUCTION

Variation in the days to flowering (heading date) occurs world-wide in the wild and cultivated rice (*Oryza sativa*) varieties. Decreasing the day length will promote floral transition whereas increasing day length will extend the vegetative growth phase (Takahashi et al., 2009). The molecular genetic pathway for short-day flowering in cultivated rice is relatively well characterized (Hayama and Coupland, 2004; Izawa, 2007; Tsuji et al., 2008). Light and circadian clock signals are first received by *OsGI*, the rice orthologue of *Arabidopsis GIGANTEA*, which in turn regulates the expression *of both heading date 1* (*Hd1*) and *OsMADS51* (Hayama et al., 2003; Kim et al., 2007). Both rice *Hd1* and its *Arabidopsis* orthologue, *CONSTANS,* encode zinc-finger transcriptional activators with the *CO*, *CO*-like and *TOC1* (CCT) domains (Yano et al., 2000). *Hd1* influences *heading date 3a* (*Hd3a*) expression (Hayama et al., 2003; Yano et al., 2000; Kojima et al., 2002). *Hd3a* is a rice orthologue of *Arabidopsis flowering locus T* (*FT*) and recently, these two genes were shown to encode a mobile flowering signal (Corbesier et al., 2007; Tamaki et al., 2007). *Rice flowering locus T1* (*RFT1*) is a member of rice *FT-like* gene family. It works as a floral activator and acts redundantly with *Hd3a* (Izawa et al., 2002; Komiya et al., 2008). *OsMADS51* encodes a type I MADS-box gene and functions upstream of *Early heading date 1* (*Ehd1*) (Kim et al., 2007). *Ehd1* encodes a B-type response regulator and works as an activator of *Hd3a* independently from *Hd1* (Doi et al., 2004). For both *Ehd1* and *OsMADS51*, there are no clear orthologues found in the *Arabidopsis* genome.

More recently, functional and nonfunctional alleles of *Hd1* were shown to be associated with early and late flowering, respectively, suggesting that *Hd1* is a major determinant of variation in flowering time of cultivated rice (Takahashi et al., 2009).

Molecular markers are widely used to track loci and genome regions in several crop species, as molecular markers tightly linked to a large number of agronomic and disease resistance traits are available in major crop species (Varshney et al., 2005). For plant breeding applications, microsatellite or simple sequence repeat (SSR) markers have been recommended as markers of choice because of their co-dominant inheritance and often differ considerably in length due to variations in the number of tandem repeats within the microsatellite region

Abbreviations: SSR, simple sequence repeat, **STS,** sequence tagged-sites; **PCR,** polymerase chain reaction; ***Hd1,*** *heading date 1;* ***Hd3a,*** *heading date 3a;* ***FT,*** *flowering locus T;* ***RFT1,*** *rice flowering locus T1*; **QTL,** quantitative trait loci; **EDTA,** ethylenediaminetetraacetic acid; **CTAB,** cetyl trimethylammonium bromide; **MAS,** marker assisted selection; **SD,** short day; **LD,** long day.

Table 1. Genotypes, pedigrees, number of days to maturation and origins of the studied rice varieties.

S/N	Genotype	Parents	Days to maturation	Origin
1	Sakha 101	Giza176/Milyang79	125 - 135 days (moderate)	Egypt
2	Sakha 103	Giza 177/Suweon 349	123 days (early)	Egypt
3	Sakha 104	GZ4096-8-1/GZ4100-9-1	125 - 135 days (moderate)	Egypt
4	Giza 171	Nahda/calady 40	155 - 160 days (late)	Egypt
5	Giza 177	Giza171/Yomji No1 //Pi No4	125 days (early)	Egypt
6	Giza 178	Giza 175/Milyang 49	125 - 135 days (moderate)	Egypt
7	BL1	Norin 25 B4/Tjina	125 - 135 days (moderate)	Japan
8	HR 5824-B-3-2-3	Akiyu Daka/Suweon 310	100 days (very early)	Egypt

(Gupta and Varshney, 2000).

This study aimed to characterize the heading date *Hd1* locus using molecular markers that are derived from DNA sequences of heading date *Hd1* quantitative trait loci (QTL) in the cultivated Egyptian rice varieties. Such markers would be of great importance in marker-assisted selection for early flowering rice varieties, which on the other hand would reduce the amount of water required for rice growth specially in areas with limited water resources like Egypt and other countries in the Middle East.

MATERIALS AND METHODS

Plant material

Eight rice varieties were obtained from the Rice Research and Training Center (RRTC) at Sakha, Kafr El-Sheikh Governorate, Egypt. Out of the eight rice varieties, one variety (HR 5824-B-3-2-3) is very early heading date (100 days to maturation), two varieties (Giza 177 and Sakha 103) are early heading date (123 - 125 days to maturation), four varieties (Sakha 101, Sakha 104, Giza 178 and BL1) are moderate heading date (125 - 135 days to maturation) and one variety (Giza 171 to maturation) is late heading date (155 - 160 days to maturation) (Table 1).

DNA isolation

Rice grains were grown in plastic pots in a growth chamber (Conviron, Canada) at the Biochemistry Department, Montreal University, Canada. The temperature was 28°C during the 16 h day and 25°C during the 8 h night and the relative humidity was kept at 60%.

Miniprep DNA extractions were performed using a 2 × cetyl trimethylammonium bromide (CTAB) extraction protocol. Roughly, 0.25 g of fresh leaf tissues were ground to powder with liquid nitrogen, then transferred into 1.5 mL centrifuge tubes. To each tube, 1 mL pre-heated DNA extraction buffer (100 mM Tris-HCl pH = 8.0, 20 mM ethylenediaminetetraacetic acid (EDTA), 1.5 M NaCl, 2% (w/v) CTAB, 1% β-mercaptoethanol was added, the tubes were vortexed and incubated at 60°C for 45 min. Afterwards, a chloroform extraction was done with 24:1 chloroform: isoamyalcohol solution, followed by isopropanol (700 µl) precipitation. DNA pellets were washed two times with 70% ethanol and finally, resuspended in 50 µl dH_2O. DNAs were run in a 0·8% agarose gel to verify both quality and concentration. Gene Ruler DNA Ladder Mix (Fermentas) was used as a standard for size and concentration.

Marker analysis

Primers selection

The genomic sequence of Nipponbare-*Hd1* (Yano et al., 2000, accession AB041837 and GI: 11094202) was used to select SSRs. The Nipponbare-*Hd1* derived primers were selected using SSRIT-simple sequence repeat identification tool (http://www.gramene.org/db/markers/ssrtool). Five SSRs were found in the Nipponbare-*Hd1* sequence- two dinucleotides (AG) 5 motifs (from 518 to 527 bp and from 1230 to 1239 bp), trinucleotide repeats (ACA) 5 motifs (from 1776 to 1790 bp), (AGC) 6 motifs (from 1965 to 1982 bp) and finally, (AGG) 5 motifs (from 1381 to 1395 bp). Three pairs of primers were designed to amplify these motives (Table 2). Another sequence which is a cDNA clone S12569 (accession AB001887 and GI: 3618317) and code for a zinc finger protein was also used for SSRs identification. One SSR was found which is a (TA) 15 motifs (Table 2). A second group of four primer pairs (P108FR, P125FR, P128FR and P130FR) were selected from the publication of Monna et al. (1995). The primers selected from the previously mentioned literature were chosen based on the criteria that they mapped closely or flanked the heading date or flower time QTL or gene(s).

PCR amplification

The PCR reactions were carried out for 35 cycles. For the initial denaturation, the PCR reactions were heated at 95°C for 5 min. The conditions for each cycle were as follow: denaturation at 95°C for 30 s, annealing at 55 - 60°C (depending on the annealing temperature of each primer) for 45 s and extension at 72°C for 30 s. The final extension was carried out at 72°C for 5 min. The PCR was done in 50 µl reactions that contained 1 × PCR buffer, 2 mM $MgCl_2$, 0.2 dNTPs, 2 µl DNA template (60 ng), 1 µl (10 pmole) of each primer, 2.5 units of *Taq* polymerase (GenScript, Piscataway, NJ). The PCR reactions were performed in a Biometra T-Gradient thermocycler (Biotron, Göttingen, Germany).

Electrophoresis

The amplified PCR products were resolved on 2% agarose gels (Bioshop Canada Inc., Canada), containing 0.5 µg/ml ethidium bromide, in 1 × TAE buffer at 100 V for 90 min. Gels were visualized under UV light transilluminator and photographed. All laboratory analyses were conducted at the Department of Biochemistry, University of Montreal, Dr. Normand Brisson Molecular Biology Laboratory (Montreal, Quebec, Canada).

Table 2. Primer names, motifs, sequences, annealing temperatures and expected amplicons of the primers used in this study.

Primer name	Motif	Forward and reverse primers (5'-3')	Annealing temperature	Expected amplicon length (bp)
Hd1-agcF	(AGC)6 and (ACA)5	GCTCCCGGCCATCACCAT	58°C	441 bp
Hd1-agcR		TCTGCTCCCACAACTCCATAACC		
Hd1-agF	(AG)5	CATGGCAGGCCGCTTTGGAAC	58°C	522 bp
Hd1-agR		TGGCGCCCTTGAGCTAAATCTAA		
Hd1-aggF	(AGG)5 and (AG)5	CACAAGAGCCATGCGAGGTAGAG	58°C	294 bp
Hd1-aggR		CGGCAGTACACCACGCTCG		
Hd1-cctF	N/A	ACAGGGAGGCCAGGGTGCT	55°C	474 bp
Hd1-cctR		CTCATTTCATCTCATCACTGCTC		
S12569-cctF	(AT)15	TACAGGGAGAAGAGGAAGACGAG	55°C	356 bp
S12569-atR		GAAGCCTCATACCTCCCACAAAT		

RESULTS

Nine (5 SSR and 4 sequence tagged-sites (STS)) markers were used for genotyping the eight rice cultivars included in the present study. At least, an amplicon of the expected size was obtained for all the five SSR markers and the four STS markers. The 4 STS markers P108, P125, P128 and P130 amplified one amplicon in all eight rice varieties, 190, 190, 160 and 110 bp, respectively. Similarly, the four SSR markers (Hd1AG, Hd1AGG, Hd1CCT and S12566CCT) showed single and non-polymorphic PCR products (522, 294, 474 and 356 bp, respectively as shown in Table 2) in all eight rice cultivars. In contrast, the SSR marker Hd1AGC was polymorphic and gave three amplicons of different sizes 441, 490 and 620 bp as shown in Figure 1.

Among the eight Egyptian rice cultivars, the number of alleles varied from one to three alleles per marker. Marker Hd1AGC amplified three PCR fragments (alleles) of various sizes (441, 490, and 620 bp) among all rice cultivars (Figure 1). On the other hand, the rest of the markers amplified only one PCR fragment (one allele) in all cultivars (Figure 1). Only the SSR marker Hd1AGC showed variation in the PCR fragment (allele) sizes among the rice varieties. Conversely, the rest of SSR markers showed no polymorphism in the PCR fragment sizes among varieties.

Genotyping of the eight cultivars with *Hd1* heading-date linked markers showed that marker Hd1AGC amplified a unique PCR fragment 620 bp in the two early heading-date cultivars (Giza 177 and Sakha 103), while the expected PCR fragment 441 bp was amplified in two moderate heading-date varieties (BL1 and Sakha 101) and in HR 5824-B-3-2-3 (very early heading date, 100 days). A third PCR fragment of 490 bp was amplified in Giza 171 (late heading date, 155-160 days to maturation) and two moderate heading date (125-135 days to maturation) Sakha 104 and Giza 178 as well as in Sakha 103 (an early heading date variety).

DISCUSSION

One SSR marker developed in this study, Hd1AGC, showed polymorphism among eight rice varieties of different heading-date times. A unique band of 620 bp amplified in two Egyptian early flowering time rice varieties indicates that Hd1AGC could be used in the marker assisted selection (MAS) and for further rice heading date studies. However, in one of the early heading-date variety, Sakha 103, a PCR fragment 490 bp was observed that was found also in the late heading date variety, Giza 171. This could be explained as Giza 171, which is one of the parents variety, Sakha 103 (Table 1). Although the expected 441 bp PCR product allele is present in the very early heading date (100 days to maturation) variety HR 5824-B-3-2-3, it is also found in two moderate heading date varieties BL1 and Sakha 101. As Yano et al. (2000) described that *Hd1* QTL explained 67% of the genetic variation in heading date locus in rice, therefore there might be another allele having a role in promoting heading in HR 5824-B-3-2-3. It appears that QTLs of large effect are often controlled by single genes and in some cases are allelic to known major genes.

In this study, using SSR markers or STS linked to the *Hd1* heading date time QTLs in rice revealed that these markers showed low level of allelic diversity. As seen in the results, most SSR markers showed one to three different amplicons (alleles). This indicates that this region is conserved and there is low level of recombination which is true in the gene regions. Saker et al. (2005) showed that some of the Egyptian rice genotypes used in their investigation have probably originated from closely related ancestors and possess a high degree of genetic similarity. Yano et al. (2000) and Kojima et al. (2002) defined 12 kb as a candidate genomic region of *Hd1* and functionally determined the gene of the *Hd1* locus, which is allelic to *Se1* (photoperiod sensitive 1 gene, a major gene controlling the response to photoperiod) and homologue of *Arabidopsis CONSTANS* (*CO*) flowering

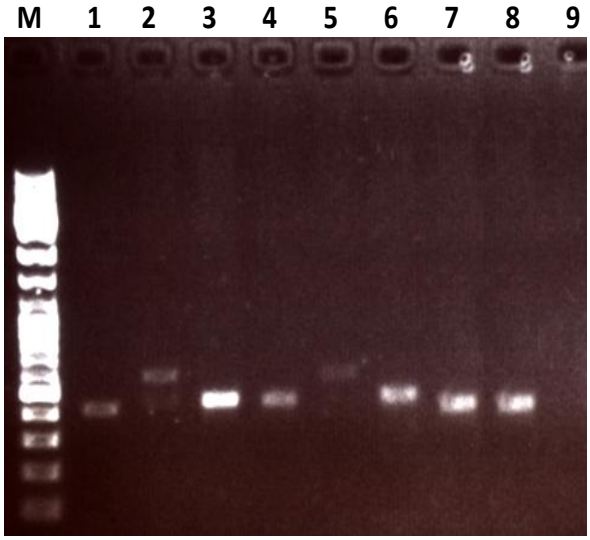

Figure 1. 2% agarose gel of amplified PCR products of eight rice varieties using Hd1AGC SSR marker. Lanes from 1 to 8 are varieties Sakha 101, Sakha 103, Sakha 104, Giza 171, Giza 177, Giza 178, BL1 and HR 5824-B-3-2-3, respectively. Lane 9 is negative control. M is DNA ladder mix.

gene.

A genetic study demonstrated that *Hd1* may function differently under short day (SD) and long day (LD) conditions to promote flowering in the SD condition and inhibit it in the LD condition (Lin et al., 2000). It is noteworthy that, under LD conditions, *Hd1* inhibits flowering of rice, whereas *CO* promotes flowering of *Arabidopsis*, suggesting that these genes may regulate the target genes in an opposite manner in LD (Yano et al., 2000).

Analysis of epistatic interactions showed that *Hd1* is epistatic to other genes that enhance photoperiod response, such as *Hd2* and *Hd3* (Lin et al., 2000; Yano et al., 2000) and suggested that *Hd1* plays a central role in the expression of photoperiod response under both SD and LD conditions. Moreover, *Hd3a* showed a high level of similarity with the *Arabidopsis FT* gene (Kobayashi et al., 1999) that promotes flowering in LD conditions. Therefore *Hd2, Hd3* and *Hd6* sequences could be used in further marker studies on Egyptian varieties to determine the amount of genetic variation in these loci and if it would be useful for rice breeding programs.

Takeuchi et al. (2001) demonstrated that rice varieties adapted to different environmental conditions are useful for discovering previously unknown allelic variation. Rice (*O. sativa*) is distributed throughout the world and there are many varieties adapted to unique environmental conditions which would provide a wide range of allelic variation. In addition, a detailed understanding of the extent and structure of crop genetic diversity is necessary for effective management and use of crop germplasm resources.

ACKNOWLEDGMENTS

The author would like to thank Dr. N. Brisson (University of Montreal, Montreal, Canada) who offered his laboratory and growth chamber facilities to perform the experiments. Special thanks also go to Dr. A. Badr (Botany Department, Tanta University, Egypt) and Dr. A. Hamwieh (ICARDA, Aleppo, Syria) for critical reading of the manuscript.

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