Türkiye Tarımsal Araştırmalar Dergisi
dergipark.gov.tr/tutad
Research Article

Turk J Agric Res
2017, 4(1): 59-64
© TÜTAD
ISSN: 2148-2306
e-ISSN: 2528-858X
doi: 10.19159/tutad.300702

Screening of *Oryza sativa* L. for *Hpt* Gene and Evaluation of *Hpt* Positive Samples Using *Houba* Retransposon-Based IRAP Markers

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Received: 20.09.2016 **Accepted:** 08.03.2017

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Abstract: Increasing world population needs to enhance agricultural production because of food starvation. Genetically modified organism (GMO) is a way to solve this problem. During gene transfers, DNA is inserted into a plant's genome in a random way. This produces spontaneous genetic changes with movement of transposable elements, and even increases variations. *Houba* was described as one of the active retrotransposons in rice. The aim of this study was to screen rice samples collected from Turkey, and analyse *Houba* retrotransposon movements with IRAP technique in transgenic ones and their controls. For this purpose, 71 different rice seeds obtained from different regions of Turkey were used for GMO analysis. All samples were screened by real time PCR to test cauliflower mosaic virus (CaMV) 35S promoter (P-35S) regions, T-NOS (nopaline synthase terminator) regions, figwort mosaic virus (FMV) regions, *bar, pat* and *Cry1ab/ac*, and *hpt* (*hygromycin resistance*) genes. *Hpt* gene was identified in 6 samples as a result of real time PCR analysis. These 6 transgenic samples with their controls were used for IRAP-PCR analysis and 0-56% polymorphism ratios were observed in analysed samples. This study is one of the first detailed experimental data of transgenic *Oryza sativa* L. samples in terms of retrotransposon-based variation.

Keywords: Genetically modified organism, *Houba,* IRAP, Real-Time PCR, rice

1. Introduction

Plant transformation technology has become an important tool for improving plants especially crop species with desirable traits (Barampuram and Zhang, 2011). Genetically modified organism (GMO) is described as an organism produced from applications of gene transfer methods. The first commercial GMO tomatoes were obtained in 1996 (The Flavor Savor TM). In 2015, agricultural biotech products were grown in 179.7 million hectares of land in the world (James, 2015). These developments have been brought the concerns of adverse effects of GMOs on human, animal health and environment. These concerns have highlighted the concept of biosecurity. For this reason, many countries set official regulations to the label of GMOs and GMO-derivative foods (Matsuoka et al., 2000; Anonymous, 2003; Miraglia et al., 2004; Vijayakumar et al., 2009). Turkey is one of these countries that published a biosafety law in 2010 (Anonymous, 2010a). According to this law, essentially modified crops and GMOs on the market are forbidden for use in infants- and young children-supplementary foods. For this reason, it is obligated to determine GM and GM derivative materials in food and feed products (Anonymous, 2010b). On the other hand, GM plants including Bt11, GA21, NK603, DAS1507, DAS59122, MON89034, MON40-3-2 (GTS-40-3-2), MON89788, A2704-12 have been accepted for using as feed (Anonymous, 2011a, 2011b).

Different methods are used to identify the GMOs. DNA-based methods are listed as GM screening; PCR, nested PCR, microarray and protein-based methods biosensor via ELISA (Gryson, 2010; Cheema et al., 2016; Turkec et al., 2016). Moreover, one of the most useful methods for GM screening is real-time PCR (Gryson, 2010). PCR-based detection can be used in different categories (Holst-Jensen et al., 2003). In the first category, screened regions are specific elements like promoter, terminator sequences as

CaMV, nopaline synthase terminator or genes encoding the resistance to antibiotics used as markers in selections. In the second category, detection is carried out by identification of specific-genes such as *bar*, *pat* and *CryIA(b)*. In category 3, junctions between promoter and genes, and in category 4, junction between gene and its integration locus are targeted for PCR amplification. The last one is also called event-specific which has been demonstrated in plants such as rice, wheat, and maize (Barroso et al., 2015; Zhang et al., 2015; Rao et al., 2016).

There are different DNA-based molecular markers to analyse genome dynamics, polymorphism, and even evolution in plants (Kaya et al., 2013; Poczai et al., 2013; Cakmak et al., 2015). The ubiquity, abundance, dispersion, and dynamism are LTR retrotransposons' characteristics in plant genomes. Therefore, these mobile elements are commonly studied as a molecular marker (Kalendar and Schulman, 2006; Poczai et al., 2013). IRAP (Inter-Retrotransposon Amplified Polymorphism) is one of them. In this method, PCR primers are designed as an outward direction from the conserved sequences of LTR. Therefore, internal regions between two LTRs or solo LTRs (without retrotransposon) are amplified (Kalendar et al., 1999). Transposons also cause different phenotypes; for example, in 4,000 transposon-insertion lines detected, about 140 lines showed a visible mutant phenotype (Kuromori et al., 2006). The first step for successful variation analysis with retrotransposons is to determine plant-specific retrotransposons. Our group recently described *Houba* retrotransposon as a good variation analyser in rice (Yuzbasioglu et al., 2016).

In this presented work, 71 rice samples were collected from several markets and Trakya Agricultural Research Institute in Turkey. CaMV 35S promoter regions, T-NOS regions, FMV regions, *bar, pat* and *Cry1ab/ac* and *hpt* genes were analysed to identify transgenics in all these samples by real-time PCR. In addition, genetically modified seeds with their non-transgenic controls were compared with IRAP-PCR by using *Houba* retrotransposon to determine variation.

2. Materials and Methods

2.1. Samples

71 rice samples were collected from different regions in Turkey. GM rice samples used for positive control were supplied from Assist. Prof. Dr. A. Akbudak from Akdeniz University.

2.2. DNA extraction

Genomic DNAs were isolated from rice samples according to Pervaiz et al. (2011) protocol. The quantities of DNAs were measured by Nanodrop (Thermo Scientific, 2000c) and qualities were controlled in 1% agarose gel electrophoresis.

2.3. GM detection

Primers and probes of *hpt* gene were designed using GenBank (accession number K01193.1) and GenScript Real-time PCR (TaqMan) primer design programme (https://www.genscript.com/ssl-bin/app/primer). The nucleotide sequences of primers and probes were shown in Table 1.

2.4. PCR conditions for GM analysis

PCR was carried out in a thermal cycler (Agilent Mx300P). PCR assays were performed in a final volume of 25 µl with 12.5 µl of PCR mix and 7.5 µl Oligo mix and 100 ng of genomic DNA for 35S, NOS, FMV, *bar, pat* and *Cry1ab/ac* screening (Eurofins). In *hpt* PCR assays for monitoring, the reaction mixture (25 µl) contained 12.5 µl of PCR master mix (Agilent Technologies), 200 mM of each primer and probes and 100 ng of genomic DNA. PCR conditions were as follows: initial denaturation at 94°C for 10 minutes, 45 cycles of denaturation at 95 °C for 15 seconds, annealing at 60° C for 1 minutes, for *hpt* gene screening and 94°C for 10 minutes, 45 cycles of denaturation at 95°C for 15 seconds, annealing at 60° C for 1.5 minutes for 35S, NOS, FMV, *cry1ab/ac*, *bar* and *pat* screening.

2.5. IRAP analysis

After GM detection, transgenic rice samples' gDNAs were used for IRAP-PCR analysis to investigate *Houba* retrotransposon movements.

Table 1. The *Hpt* gene primers' and probe's nucleotide sequences

Hpt R	5'- ATGCAAAGTGCCGATAAACA-3'
Hpt L	5-'ATGTCCTGCGGGTAAATAGC-3'
Hpt P	5'-FAM- TGCGCCGATGGTTTCTACAAAGATC-TAMRA-3'

Primer sequences designed by using IDT (Integrated DNA Technologies) were shown in Table 2. IRAP-PCR was performed in a total volume of 20 µL, containing 20 ng template DNA, 10 nmol forward and reverse primers and SapphireAmp Fast PCR Master Mix (Takara, RR350A). Primer dimer or other contaminations were checked by using no template control (negative control). In this control, the PCR contents were the same as in IRAP-PCR, but without template (water was used instead of template). The PCR conditions were as follows: initial denaturation at 95 ºC for 2.5 minutes, followed by 30 cycles at 94 ºC for 30 seconds, 51 ºC for 30 seconds, 72 ºC for 2.5 minutes and the reaction was completed with a cycle of final extension at 72 ºC for 7 minutes. The PCR products were resolved in a 8% polyacrylamide (29:1 Acrylamide:Bis) gel electrophoresis (Bio-Rad, Protean II xi Cell) at 150 V for 8 h in 1X TBE buffer (pH= 8.0). A molecular weight marker (Thermo Scientific, SM0331) was also loaded to determine the sizes of the PCR fragments. After, the gel was visualised on UV transilluminator, photographed and used for data analysis.

2.6. Data analysis

The well-resolved bands were scored as a binary value, '1' for presence and '0' for absence of bands. The binary matrix (1/0) was used to calculate the similarity using Jaccard's coefficient (Jaccard, 1908).

Table 2. The *Houba* retrotransposon primer sequences used for IRAP analysis

Houba-F	5'-CTTCGAGTGGGCTAAGGCCC-3'
Houba-R	5'-GTTTCGACCAAGCAGCCGGTC-3'

3. Results

3.1. GM analysis

In this study, rice samples were investigated for GM elements of 35S, NOS, FMV elements and also *bar*, *pat*, *cry1ab/ac* and *hpt*. As a result of analysis, no PCR amplification observed in 35S, NOS, FMV and *cry1ab/ac, bar* and *pat* experiments. On the other hand, we found that 6 samples of 71 rice seeds were transgenic because of *hpt* gene amplification (Figure 1).

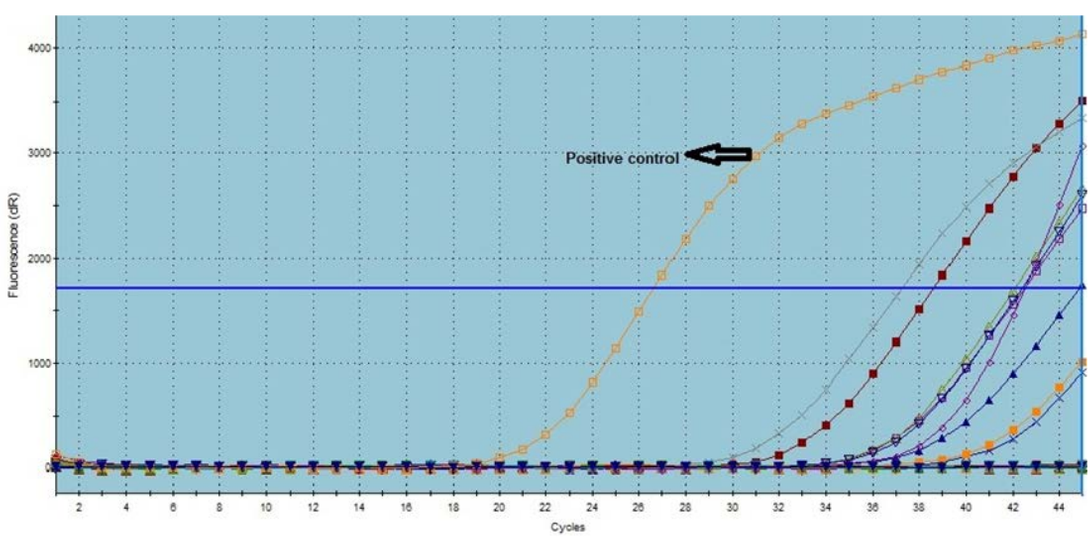

Figure 1. The *Hpt* gene real time amplification results. Orange line is positive control, other lines are samples

3.2. IRAP analysis

After determination of transgenic samples, these seeds with non-transgenic controls were analysed with IRAP marker to determine *Houba* retrotransposition. Six samples (hygromycin resistant), two control transgenic rice and two non-transgenic rice seeds were investigated using *Houba* retrotransposon by IRAP-PCR (Figure 2).

Retrotransposon bands showed different profiles among samples with the length between

250 and 1500 bp. IRAP-PCR analysis showed that there were polymorphic bands among samples. Polymorphism rates were 0-56% among samples. Moreover, polymorphism ratios were 0-25% for between transgenic samples, 19% for control transgenic rice and 19% for non-control transgenic rice (Table 3).

4. Discussion

The impact of the insertion of DNA through genetic engineering is probably paralleled by the movement of transposable elements. Transposable elements can be found in the genomes of all plants. Rice genome project results showed that 35% of rice genome is consist of transposons (TEs). IRAP marker system can be a useful tool for investigating rice breeding (Kalendar et al., 1999). However, a retrotransposon based marker, like IRAP, has not been used for the identification of variation in transgenic rice until now.

Real time PCR is widely used for GMO analysis to identify the presence of most commonly integrated DNA elements in GMOs, and also used event-specific methods provided by the GMO developers (Broeders et al., 2012). In Turkey, the screening process is carried out by Real-Time PCR for only 35S, NOS, and FMV genetic elements. However, there are so many GM events that have different genetic elements rather than 35S, NOS, and FMV. Moreover, *hpt* gene provides resistance to aminoglycoside antibiotics such as hygromycin B (Van den Elzen et al., 1985). This antibiotic is much more effective than kanamycin for the selection of transformed rice tissues (Christou and Ford, 1995). Zuraida et al. (2013) studied with *hpt* gene in *Agrobacterium*-mediated genetic transformation of rice and concluded that hygromycin B is a suitable selection agent and selective marker for genetic transformation. In addition to *Agrobacterium*-mediated transformation, zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) 9 (CRISPR/Cas9) have been commonly used for plant genome editing (Baltes and Voytas, 2015;

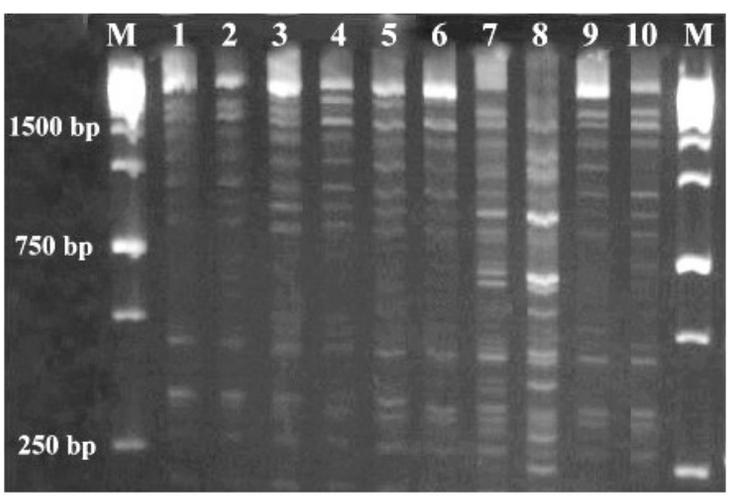

Figure 2. *Houba* retrotransposon variation analysis with IRAP-PCR
Transgenic rice: 1, 2, 3, 4, 5, 6. Control transgenic rice: 7, 8. Non-transgenic rice: 9, 10

Table 3. Polymorphism rates of *Houba* retrotransposon

	1	2	3	4	5	6	7	8	9	10
1	-	19	19	20	20	13	44	50	7	13
2		-	24	25	25	25	40	52	24	29
3			-	13	13	19	46	**56**	24	53
4				-	0	7	54	54	29	35
5					-	7	50	54	25	31
6						-	50	50	19	29
7							-	19	40	40
8								-	**56**	50
9									-	19
10										-

Transgenic rice: 1, 2, 3, 4, 5, 6. Control transgenic rice: 7, 8. Non-transgenic rice: 9, 10

Weeks et al., 2016). Especially CRISPR/Cas9 system has been used for genome editing in major crops such as rice (Zhang et al., 2014; Xu et al., 2015) maize (Feng et al., 2016) and wheat (Shan et al., 2014).

In this study, 35S, NOS, FMV, elements and *bar, pat, cry1ab/ac* genes were not detected but *hpt* gene was found in 6 samples by using Real-Time PCR analysis in total 71 rice seeds collected from different sources. Results showed that the GMO screening process could be modified to increase screening range of genetic elements or genes in Turkey. *Houba* retrotransposon movements in transgenic rice with their controls were investigated and 0-56% polymorphism ratios were observed among all samples. In addition, we also found that there were 0-25% for between transgenic samples, 19% for control transgenic rice and 19% for non-control transgenic rice.

In addition to important role of transposon in genome structure and gene functions, information about this issue is still at beginning level (Schnell et al., 2015). In this study, effects of transformation in GM rice on transposon movements were investigated. For this purpose, retrotransposon movements were compared in GM rice and non-GM rice seeds by using *Houba* retrotransposon specific IRAP-PCR. Results are expected to contribute GMO screening by using *hpt* gene and determination of transformation results after gene transfers at molecular level.

Acknowledgements

This work was supported by Scientific Research Projects Coordination Unit of Istanbul University. Project numbers: 40028 and 20366.

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