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*MORPHOLOGICAL AND MOLECULAR IDENTIFICATION FOR SIX PROMISING HULL-LESS BARLEY (*HORDEUM VULGARE* L.) GENOTYPES

BY

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ABSTRACT

Identification of plant genotypes is an important process to registries the plant cultivars, protect breeder's right, maintain the genotype genetic purity, and perform the field inspection as a supportive method to seed analysis, intern protect seed industry. Therefore, this study aimed to evaluate six hull-less barley (*Hordeum vulgare* L.) genotypes during 2005/2006 and 2006/2007 growing seasons using morphological description and Random Amplified Polymorphic DNA (RAPD) technique as a molecular method. The UPOV guidelines for barley variety identification were used to prepare the morphological description. The morphological characters indicated the presence of great differences at vegetative, flowering and maturity stages. Phenol reaction was also used to determine the physiological based on seed coloration quality. Moreover, the RAPD analysis revealed greater differences among various genotypes. It was found that DNA markers with specific molecular weight characterized each genotype. Therefore, fingerprinting is considered an important tool for plant cultivars identification. It was suggested to combine the genetic fingerprinting to the morphological description to obtain more accurate identification for both registration and protection purposes.

INTRODUCTION

In Egypt, barley (*Hordeum vulgare* L.) is the main crop grown in large scale in rain-fed areas. It was adapted long time ago to survive and grow satisfactorily under adverse conditions especially drought stress where water requirements for production of unit weight of grains is less than the other cereals. Genotype identification is an important issue in agriculture to protect investments for crop improvements through breeder's right. Discrimination among genotypes as well as to assess genetic purity of seed samples will help seed production industry, intern seed producers and farmers to concentrate their efforts on high quality seed. Generally, hull-less barley now is gaining renewed interest as food component because of its soluble dietary fibers and ß glucan content in particular as compared with other cereals. With this respect, El-Sayed (2002) compared 11 hull-less barley genotypes with Sakha 69 wheat cultivar regarding grain protein percentage, total and soluble ß glucan contents. These three chemical traits of all hull-less barley exceeded those of Sakha 69 wheat cultivar. In addition, the high ß glucan content of its grain is beneficial against hypercholesterolemia Atanassov *et al.*, (1999). The hull-less genes have been reported

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not to affect visible morphological and genetic characteristics, they also do not decrease yield except for the loss of hulls at harvest Heen *et al.*, (1991). To license any field crop seeds to be traded in Egypt and most countries, the crop variety should be registered and tested at least for distinctness, uniformity and stability (DUS). The variety should prove its distinctness and that it can be distinguished from other varieties of the same crop with one or more characteristics Copeland and McDonald (1995). The variety testing includes morphological and molecular profiling techniques to identify the requested genotype UPOV (1994) and Cooke (1999).

PCR-based DNA analysis such as RAPD (Williams *et al.*, 1990) has been already proven valuable in genetic analysis. The amplified DNA fragments represent anonymous regions distributed randomly throughout the genome of the organism and provide a fingerprint of the plants being investigated (Rossetto *et al.*, 1997). The advantages of this technique are its simplicity, speed, and requirement for only small amounts of genomic DNA. DNA markers obtained by RAPD-PCR give a much higher degree of polymorphism and stability.

Many investigators employed RAPD technique to assess genetic fingerprints in a wide range of plants (Aly *et al.*, 2000; Abdel-Tawab *et al.*, 2001; Cheng *et al.*, 2002 and Zhuravlev *et al.*, 2003). They reported that polymorphisms detected by arbitrarily primed PCR are useful molecular markers in population studies.

The objectives of this study were to (1) identify the actual description of six promising hull-less barley lines, (2) determine the distinguishable characteristics of each line, and (3) granting and securing plant breeder's right (PBR).

MATERIALS AND METHODS

Plant Materials:

Seed samples of six hull-less barley promising lines were provided by the Egyptian French Project for "Enhancement of Hull-less Barley Production Based on Drought Tolerance" in Egypt. These were: (1) LHB2005/1, (2) LHB2003/2, (3) LHB2005/3, (4) LHB2005/8, (5) LHB2005/10, and (6) LHB2005/12. They were exposed to selection procedures through the project activities to identify the genotypes suitable to registered and cultivated in the different ecological regions in Egypt.

Morphological Characterization:

Field experiments were conducted at Gemmeiza Agricultural Experimental Station in two consecutive seasons (2005/2006 and 2006/2007). Randomized Complete Blocks Design was used for this study. Sowing dates in the two seasons were in the first week of December. Data was collected two weeks periodically using the UPOV (The International Union for the Protection of New Varieties of plants) barley descriptor form no. TG/19/10 of 1994, which includes 29 morphological characteristics in addition to three more characteristics are seed shape distinguishing characteristic, phenol test reaction and 1000 seed weight. The Decimal Code for the Growth Stages of Cereals, 1987 was used to standardize the growth stages of barley plants during morphological description and identification.

Phenol Test:

A 1% solution of phenol was used to identify the hull-less barley six lines. 100 seed from each genotype was soaked in distilled water for 16 hours, after draining the water; the seeds are placed on two layers of filter paper and soaked in the 1% phenol solution. After one hour, the different genotype seeds will have varying degrees of coloration.

Seed Shape and Weight:

The seed shape and weight were done according to the UPOV guidelines.

DNA extraction

DNA was extracted from 100 mg of young leaves for each line using mi-Plant Genomic DNA Isolation Kit (metabion). The concentration and purity were determined by spectrophotometer.

RAPD-PCR analysis

RAPD analysis was carried out according to Williams *et al.*, (1990) the primers used were 10-mer oligonucleotide. Ten primers were selected as potentially useful. The codes and sequences of the used primers are shown in Table (2).

PCR reactions were optimized and mixtures (25 µl total volume) were composed of dNTPs (200 µM), Mg Cl2 (1.5 mM), 1x buffer, primer (0.2 µM), DNA (50 ng), Taq DNA polymerase (2 units). Amplification was carried out in a Thermo Cycler (PTC 200) programmed for 94 °C for 3 min (one cycle); followed by 94 °C for 30 sec, 36 °C for 1 min and 72 °C for 2 min (36 cycle); 72 °C for 10 min (one cycle), then 4 °C (infinitive). Amplification products (15 µl) were mixed with 3 µl loading buffer and separated on 1.3% agarose gel and stained with 0.5 µg/ml ethidium bromide, and visualized under ultraviolet light and photographed. DNA fragment sizes were determined by comparisons with the 100 bp+ 1.5 kb DNA Ladder marker.

Data analysis

The results of RAPD analysis and morphological traits were entered in a computer file as binary matrices where 0 stands for the absence of a band and 1 stands for the presence of a band in each individual sample. Similarity coefficients were calculated according to Dice matrix (Nei and Li, 1979). Construction of the dendrogram tree was performed using the unweighted pair group method based on arithmetic mean (UPGMA) as implemented in the SPSS program version 10.

RESULTS AND DISCUSSION

Morphological traits

Results in Table 1 showed the morphological description of the six lines. These results showed some wide variations among some characteristics that can be used to differentiate among these genotypes i.e. characteristics numbers: 1, 3, 4, 7, 8, 9, 10, 11, 12, 15, 16, 17, 19, 24, 25, 29, 30 and 32. These characteristics are manifested in the vegetative, maturity, and after harvest stages. The phenol test was used to aid in the identification of these six genotypes depending on phenol oxidases activity. The enzymes present in the pericarp, aleurone and other seed live structures oxidize the

phenols. Because of enzymatic oxidation activities, dark, colored, insoluble pigments or melanines are formed. The amounts and kinds of the various oxydase type enzymes present in the different tested genotypes cause varying degrees of coloration in the pericarp. This test contributed in the identification of the six genotypes, or at least to group these genotypes to groups, each has the same or close reaction to the phenol test, which has been shown in Table (1) characteristic No. 29. The weight of 1000 kernel and seed shape can offer some contribution in identifying these genotypes. According to UPOV description presented in Table (2), hull-less barley lines nos. 1, 2, 3, and 4 have one or more distinguished characters could be used to identify there lines. Line 1 showed unique figure for characters nos. 25 and 30. Line 2 is distinguished in character nos. 3, 4, 8, and 9. Line 3 is distinguished in character nos. 12, and 16 while line 4 is distinguished in character nos. 7 and 25.

RAPD-PCR profile

The ten primers used in this study displayed marked amplification with distinct bands. The RAPD markers generated by these primers revealed characteristic profiles for each line in terms of number and position of RAPD bands (Table 2 and Fig. 1). A total number of 90 DNA bands were detected as generated by the 10 random primers for the six lines used in the present study, in which 85 (94.4%) were polymorphic bands. However, 5 bands were common (monomorphic) for all lines. The lowest number of polymorphic bands was detected for primer B15 (zero out of 3 amplified band), while the largest number of polymorphic bands was detected for primer B20 (13 out of 13 amplified bands). Line-specific markers generated from RAPD-PCR analysis are shown in Table (2). Forty-four out of 90 RAPD-PCR bands were found to be useful as line-specific markers. The largest number of RAPD-PCR markers was scored for line 1 (23 markers), while the lowest (1 marker) was scored for line 4. No negative specific markers were scored for the absence of unique bands for a given line. In the meantime, primer B02 generated the largest number of RAPD-PCR line-specific markers (9 markers), while the primer B08 produced the lowest number of RAPD-PCR line-specific markers (1 marker). On the other hand, primer B15 did not produce any specific markers. In conclusion, nine out of the 10 primers used allowed enough distinction among the lines under study. These line-specific markers can be used in subsequent experiments to detect molecular markers for polymorphic genes with economic importance among these and other lines. In this respect, El-Sayed et al. 2002 (A & B) followed the same methodologies with different barley genotypes and reported similar conclusion. Hahn *et al.*, (1995) reported that even though RAPD markers are useful for grouping inbred lines with different genetic backgrounds, RFLPs are better for determining the genetic relatedness between lines. Beaumont *et al.*, (1996) reported that the RAPD technique was found to be a powerful method to provide improved probes coverage on a previously created RFLP map and to locate markers linked to chromosomal regions of interest. Although RAPD analysis is quick and well adapted for the efficient non-radioactive DNA fingerprinting of genotypes (Thorman *et al.*, 1994), problems with reproducibility of amplification and with scoring of error data have been reported for RAPDs (Demeke *et al.*, 1997 and Karp *et al.*, 1997). Powell *et al.*, (1996) and Pejic *et al.*, (1998) found the lowest correlations among RAPDs and other marker system (SSRs, AFLPs, and ISSRs).

Table (1): Morphological identification of the six hull-less barley genotypes according to UPOV descriptors.

Characteristics	Stage	Degree	LHB 2005/1	LHB 2003/2	LHB 2005/3	LHB 2005/8	LHB 2005/10	LHB 2005/12
1-Growth habit	VG (25-29)	Erect (1) Semi erect (3) Intermediate (5) Semi prostrate (7) Prostrate (9)	3	3	3	5	3	5
2-Lowest leaves: hairiness of leaf sheaths	VG (25-29)	Absent (1) Present (9)	1	1	1	1	1	1
3-Flag leaf: anthocyanin coloration of auricles	VG (45-49)	Absent (1) Present (9)	1	9	1	1	1	1
4-Flag leaf: intensity of anthocyanin coloration of auricles	VG (45-49)	Very weak(1) Weak (3) Medium (5) Strong (7) Very strong (9)	1	3	1	1	1	1
5-Plant: frequency of plants with recurved flag leaves	VG (47-51)	Absent or very low (1) Low (3) Medium (5) High (7) Very high (9)	3	3	3	3	3	3
6-Flag leaf: glaucosity of sheath	VG (50-60)	Absent or very weak(1) Weak (3) Medium (5) Strong (7) Very strong (9)	1	1	1	1	1	1
7-Time of ear emergence (first spikelet visible on 50% of ears	VG (50-52)	Very early (1) Early (3) Medium (5) Late (7) Very late (9)	7	5	7	3	5	5
8- Awns: anthocyanin coloration of tips	VG (60-65)	Absent (1) Present (9)	1	9	1	1	1	1
9-Awns: intensity of anthocyanin coloration of tips	VG (60-65)	Very weak(1) Weak (3) Medium (5) Strong (7) Very strong (9)	1	3	1	1	1	1
10-Ear: glucosity	VG (65-75)	Absent or very weak(1) Weak (3) Medium (5) Strong (7) Very strong (9)	3	3	1	1	1	1
11-Ear: attitude	VG (70)	Erect (1) Semi-erect (3) Horizontal (5) Semi-recurved (7) Recurved (9)	3	5	3	3	5	3

Table (1): cont.

Characteristics	Stage	Degree	LHB 2005/1	LHB 2003/2	LHB 2005/3	LHB 2005/8	LHB 2005/10	LHB 2005/12
12-Plant: length (stem, ear and awns)	M (80-92)	Very short (1) Short (3) Medium (5) Long (7) Very long (9)	7	7	5	9	9	7
13-Ear: number of rows	VS (80-92)	Two (1) More than two (2)	2	2	2	2	2	2
14-Ear (Shape)	VS (80-92)	Tapering (3) Parallel (5) Fusiform (7)	5	5	5	5	5	5
15-Ear: density	VS (80-92)	Very lax (1) Lax (3) Medium (5) Dense (7) Very danse (9)	7	7	7	5	5	7
16-Ear length (excluding awns)	M (80-92)	Very short (1) Short (3) Medium (5) Long (7) Very long (9)	7	7	3	5	5	5
17-Awn: length (compared to ear)	VS or M (80-92)	Short (3) Medium (5) Long (7)	3	3	3	7	7	7
18-Rachis (length of first segment)	VS (92)	Short (3) Medium (5) Long (7)	5	5	5	5	5	5
19-Rachis (curvature of first segment	VS (92)	Absent or very weak(1) Weak (3) Medium (5) Strong (7) Very strong (9)	3	3	5	3	3	5
20-Sterile spikelet: attitude (in mid-third of ear)	VS (92)	Parallel (1) Parallel to weakly divergent (2) Divergent (3)	2	2	2	2	2	2
21-Median spikelet: length of glume and its awn relative to grain	VS (92)	Shorter (1) Equal (2) Longer (3)	1	1	1	1	1	1
22-Grain (rachilla hair type	VS (80-92)	Short (1) Long (2)	1	1	1	1	1	1
23-Grain (husk)	VS (92)	Absent (1) Present (9)	9	9	9	9	9	9
24-Grain (anthocyanin coloration of nerves of lemma	VS (80-85)	Absent or very weak(1) Weak (3) Medium (5) Strong (7) Very strong (9)	1	3	1	1	1	1
25-Grain (spiculation of inner lateral nerves of dorsal side of lemma	VS (92)	Absent or very weak(1) Weak (3) Medium (5) Strong (7) Very strong (9)	5	3	3	7	3	3

Table (1): cont.

Characteristics	Stage	Degree	LHB 2005/1	LHB 2003/2	LHB 2005/3	LHB 2005/8	LHB 2005/10	LHB 2005/12
26-Grain : hairiness of ventral furrow	VS (92)	Absent (1) Present (9)	1	1	1	1	1	1
27-Grain: disposition of lodicules	VS (92)	Fronting (1) Clasping (2)	1	1	1	1	1	1
28-Kernel: color of aleurone layer	VG (85-87)	Whitish (1) Weakly colored (2) Strongly colored (3)	1	1	1	1	1	1
29-Phenol Reaction	M	Weak (1) Slight (3) Moderate (5) Strong (7) Very strong (9)	3	7	7	3	3	7
30-1000 seed wt (gm)	M	Gm	40.99	38.50	43.02	48.03	44.27	44.33
31-Seasonal type	VG	Winter type (1) Alternative type (2) Spring type (3)	3	3	3	3	3	3
32-Grain (shape)	M	Length(long-L) Medium(M) Width(Slim-S) Medium(M) Wide (W)	M,M	M,M	M,M	M,W	M,W	M,W

In conclusion, when we use another PCR-based marker technique such as ISSR, SSR, and AFLP, we will obtain higher information content and consequently higher distinguishable among the used genotypes.

Table (2): Summary of data obtained by RAPD analysis for the six hull-less barley genotypes.

Primer	Sequence 5⟶3	TAB	PB	Genotypes 1 AB	1 SM	2 AB	2 SM	3 AB	3 SM	4 AB	4 SM	5 AB	5 SM	6 AB	6 SM	TSM
A11	CAATCGCCGT	10	10	4	2	3	0	3	2	2	0	2	0	3	1	5
B01	GTTTCGCTCC	12	12	6	4	3	1	3	0	3	0	3	0	3	2	7
B02	TGATCCCTGG	12	12	3	3	3	2	3	3	3	1	3	0	3	0	9
B04	GGACTGGAGT	11	11	7	4	4	1	4	0	3	0	4	0	3	1	6
B08	GTCCACACGG	3	1	3	1	2	0	2	0	2	0	2	0	2	0	1
B10	CTGCTGGGAC	12	12	6	3	5	1	4	0	3	0	4	1	3	0	5
B13	TTCCCCCGCT	7	7	4	2	3	0	3	0	2	0	2	0	2	0	2
B14	TCCGCTCTGG	7	7	3	2	2	0	2	0	2	0	2	0	2	1	3
B15	GGAGGGTGTT	3	0	3	0	3	0	3	0	3	0	3	0	3	0	0
B20	GGACCCTTAC	13	13	5	2	3	2	4	0	4	0	4	1	4	1	6
Total		90	85	44	23	31	7	31	5	27	1	29	2	28	6	44

TAB= Total amplified bands, PB= Polymorphic bands, TSM= Total specific markers AB= amplified band and SM= specific marker

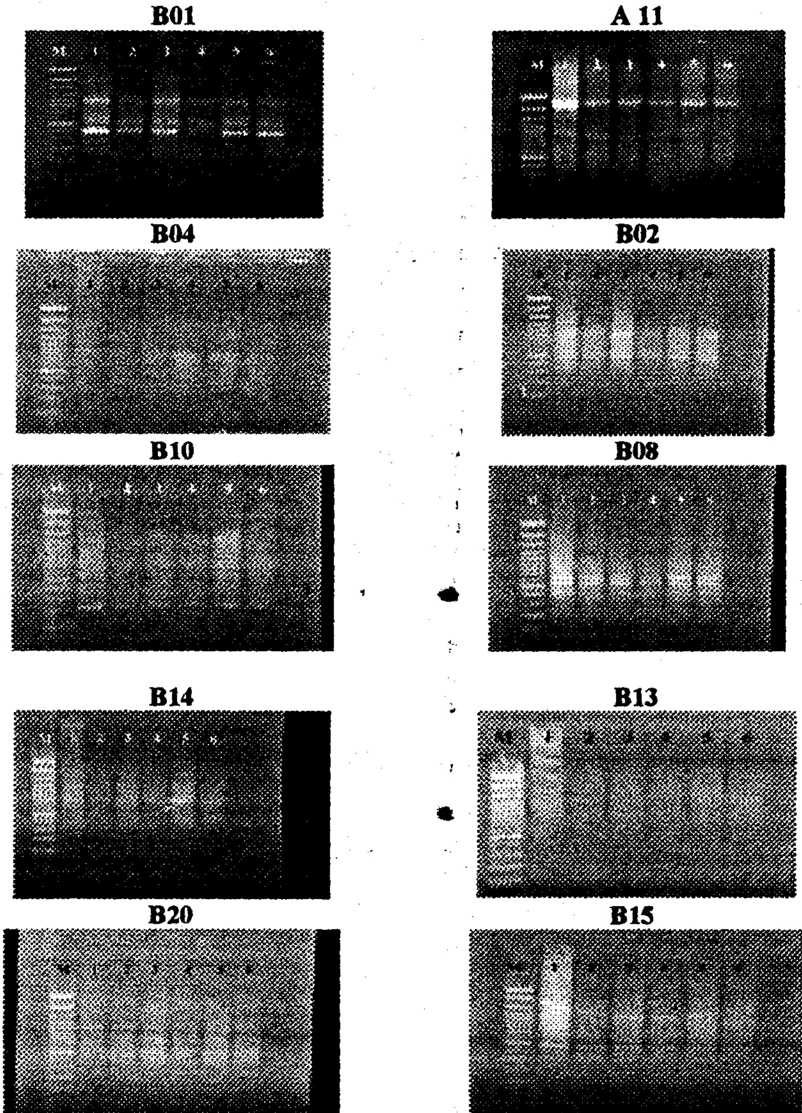

Figure (1): Agarose gel (1.2%) in TAE buffer stained with ethidium bromide showing RAPD-PCR polymorphism of DNA for six hull-less barley genotypes (Lanes 1-6, LHB2005/1, LHB2003/2, LHB2005/3, LHB2005/8, LHB2005/10 and LHB2005/12, respectively) using random primers. M refers to 100 bp DNA Ladder plus.

The genetic distance among lines

The similarity indices and the dendrogram tree among lines utilizing RAPD markers are shown in Table (3) and Fig. (2), respectively. A high relationship was scored between line 4 and line 5 (similarity index 68%), while the lowest relationship was scored between line 1 and line 6 (similarity index 28%). From the dendrogram tree, line 1 was grouped in one cluster, wile the rest of lines were grouped in another cluster.

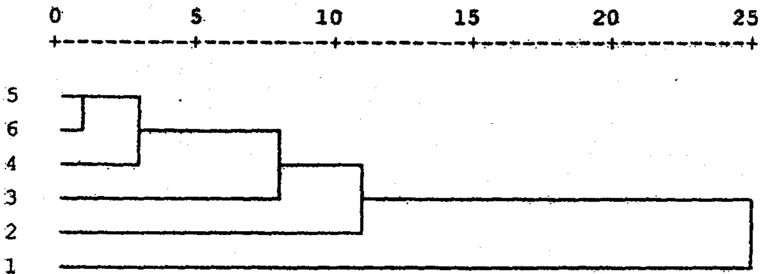

Figure (2): Dendrogram of the genetic distances among the six inbred lines of hulls barley based on RAPD analysis and morphological traits.

Table (3): Similarity matrix among the six lines based on combined analysis of RAPD analysis and morphological traits.

Line	1	2	3	4	5
1. LHB2005/1					
2. LHB2003/2	0.39				
3. LHB2005/3	0.39	0.44			
4. LHB2005/8	0.29	0.38	0.54		
5. LHB2005/10	0.29	0.42	0.52	0.68	
6. LHB2005/12	0.28	0.35	0.51	0.61	0.65

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التوصيف المورفولوجي والجزيئي لستة تراكيب وراثية مبشرة من الشعير العاري *

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يعتبر توصيف التراكيب الوراثية النباتية عملية هامة جدا لتسجيل الأصناف النباتية، حماية حق المربي، المحافظة علي النقاوة الوراثية للتراكيب الوراثية، وإجراء عملية التفتيش الحقلي، وبالتالي حماية صناعة التقاوي. هذا سوف يساعد منتجي التقاوي علي تركيز جهودهم لإنتاج تقاوي عالية الجودة. لذلك قامت هذه الدراسة بتقييم ستة تراكيب وراثية من الشعير العاري خلال موسمي ٢٠٠٥/٢٠٠٦ ، ٢٠٠٦/٢٠٠٧ بواسطة طرق التوصيف المورفولوجي والجزيئي لكي يتم تسجيلها وحمايتها. استخدم في التوصيف المورفولوجي نموذج UPOV وكذلك استخدم تكنيك RAPD-PCR في التوصيف الجزيئي للتعرف على الاختلافات الوراثية. أوضحت نتائج التوصيف المورفولوجي وجود اختلافات معنوية في مراحل النمو الخضري، التزهير، النضج واختبار الفينول. كما أظهرت نتائج تحليل ال RAPD اختلافات جوهرية بين التراكيب الوراثية المختلفة، حيث وجدت دلائل DNA بأوزان جزيئية معينة لكل تركيب وراثي. لذلك تعتبر البصمة الوراثية أداة هامة لتوصيف التراكيب الوراثية. من هذه النتائج يمكن الإشارة إلي أهمية الاعتماد على التوصيف المورفولوحي و الجزيني للوقوف على الاختلافات بين التراكيب الوراثية للحصول على معلومات دقيقة يمكن الاستفادة منها عند تقديم أي من هذه التراكيب الوراثية للتسجيل.

- ورقة علمية مستخلصة من تقرير المشروع المصري الاوروبى للتنمية المستدامة لإنتاج الشعير بالاراضى المطرية.