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Full Length Research Paper

Molecular characterization of barley (*Hordeum vulgare* L.) genome for drought tolerant cultivars selection

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The present work aimed to select drought tolerant barley (*Hordeum vulgare* L.) cultivars through identification of stress genes responsible for drought tolerance. Several barley genotypes were tested for drought resistance using specific molecular markers, nine out of all the genotypes were chosen for this study; five out of them were drought resistant (ALEXES, Giza 121, Giza 122, Giza 123 and Giza 128) and four were drought sensitive (Jazan, Qassim, Giza 123 and Giza 2000). The presence of late embryogenesis abundant (*LEA*) proteins genes was correlated with the drought tolerance in the studied cultivars; hence, this gene confers drought tolerance trait. Drought stress responses such as reduced plant height, number of leaves, tillers and leaf area were noted in all genotypes under water stress conditions as compared to the normally irrigated ones. In order to characterize their genomes, the studied barley cultivars were further analyzed using 10 simple sequence repeats (SSR) and 11 inter-simple sequence repeats (ISSR) primers. The resulted SSR (19 markers) and ISSR (39 markers) were analyzed using NTSYS-pc program for addressing the phylogenetic relationships of the studied genotypes.

Key words: Cultivated barley, drought tolerance, late embryogenesis abundant (*LEA*), simple sequence repeats (SSR), inter-simple sequence repeats (ISSR).

INTRODUCTION

Barley is one of the most important cereal crops grown in many developing countries, where it is often subjected to extreme drought stress that significantly affects production. More recently, high-throughput screening techniques have been used to monitor the expression of genes that respond to abiotic stresses (Walia et al., 2006; Talamè et al., 2007). Barley is characterized by its drought tolerance and subsequently grows in many countries characterized by extreme water deficiency during the dry season (Ceccarelli, 1994). It is known that under water deficit conditions, plants have reduced shoot growth in order to limit transpiration (Thompson et al., 2007).

Drought tolerance is a key trait for increasing and stabilizing barley productivity in dry areas worldwide. Identification of the genes responsible for drought tolerance in barley (*Hordeum vulgare* L.) will facilitate understanding of the molecular mechanisms of drought tolerance, and also facilitate the genetic improvement of barley through marker-assisted selection or gene transformation (Guo et al., 2009). Stanca et al. (1996) found that a protein of about 14 kD was encoded as a result of drought response in barley. Proline accumulation has been reported in different plant species (Choudhary et al., 2005; Shao et al., 2006; Zhang et al., 2007; Haudecoeur et al., 2009; Yang et al., 2009) and a protective role for this amino acid in plant stress adaptation has been strongly suggested (Verbruggen and Hermans, 2008). Nevertheless, a correlation between proline accumulation and abiotic stress is not always so apparent and is not correlated with salt tolerance in barley (Chen et al., 2007, Widodo et al., 2009).

Water shortage during grain development can lead to poor seed set and premature grain abortion. Drought is

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one of the main environmental constraints to agricultural productivity worldwide. The mechanisms of drought tolerance in plants through molecular and genomics approaches via number of genes that respond to drought stress at the transcriptional level have been reported (Hazen et al., 2005; Talamè et al., 2007). Furthermore, all previous studies were conducted on seedlings, whereas drought stress at the reproductive stage may have much more effect on grain yield than drought at the vegetative stage. Therefore, the analysis of gene expression for drought tolerance during the reproductive stage may provide further insight into the molecular mechanisms of drought tolerance in barley (Ceccarelli et al., 2004, 2007).

The aim of the current study was to show drought resistance genes and molecular markers correlated to drought tolerance in the cultivated barley. Molecular screening was done using PCR amplification of late embryogenesis abundant (*LEA*) gene, simple sequence repeats (SSR) and inter-simple sequence repeats (ISSR) markers which are known to be correlated with drought tolerance in order to use this machinery for screening other barley genotypes.

MATERIALS AND METHODS

Plant material and growth conditions

The current study was conducted in a controlled growth chamber at the Faculty of Science, King Abdulaziz University, Jeddah, Kingdom of Saudi Arabia. Nine barley genotypes differing in drought tolerance were chosen for this study. These genotypes were Jazan, Qassim, Alexes, Giza 121, Giza 122, Giza 123, Giza 124, Giza 128 and Giza 2000. Plant samples were divided into 2 groups: control plants (well irrigated) and water stressed plants (by withholding irrigation) until flowering and seed setting. The morphological features: plant height, leaf number, leaf area and number of tillers were noted in all cultivars in normal and water stress conditions.

DNA extraction

DNA was extracted from fresh frozen leaves of 14 days old seedlings using Bioflux DNA extraction kit, Hangzhou Bioer Technology Co. Ltd.

LEA gene amplification

Two specific PCR primers for *LEA* gene was constructed by Metabion International AG, D-82152 Martinsried, Germany. The sequence of these primers is as follows: *LEA* forward: 5-ATGGCTCGCTGCTCTTACTC-3, *LEA* reverse: 5-TCAGTGAGAGGATCGATTGAAC-3.

DNA amplification was carried out in 25 µl reaction mixture containing 50 ng genomic DNA in 5 µl, 2.5 µl 10X PCR buffer, 2 µl mM $MgCl_2$, 2 µl of each of the forward and reverse primers (10 PM), 2.5µl of 0.2 mM dNTPs (from Promega), 0.5 µl *Taq* DNA polymerase (GoTaq Flexi DNA polymerase from Promega) and 8.5 µl distilled deionized water. PCR amplification was carried out as described by Temnykh et al. (2000) as follows: 5 min at 94°C followed by 35 cycles of 1 min at 94°C, 1 min at 55°C and 2 min at 72°C, with a final extension of 5 min at 72°C. The amplification product was resolved on 1.5% agarose gel against 1 kb DNA ready load ladder from Solis BioDyne, Riia 185a, 51014 Tartu, Estonia.

SSR assay

Eleven SSR primers were selected to represent the entire barley genome. These primers were constructed by Metabion International AG, D-82152 Martinsried, Germany). The primers name, sequence, chromosomal location and annealing temperature (Ta) are given in Table 1. PCR mixture and amplification condition are the same as described by Temnykh et al. (2000) and as mentioned above in *LEA* gene amplification, except Ta which is specific for each marker as shown in Table 1.

ISSR fingerprinting

Ten ISSR primers were constructed by Metabion International AG, D-82152 Martinsried, Germany for fingerprinting the studied genotypes of barley. Their names and sequences are given in Table 2. Amplification was carried out in 20 µl reaction consisting of 4 µl master mix (5x Fire Pol Master Mix from Solis BioDyne, Riia 185a, 51014 Tartu, Estonia), 2 µl 10 PM of each primer, 50 to 100 ng genomic DNA in 2 and 12 µl of sterilized distilled water. The reaction was carried out in Biorad thermocycler programmed as follows: an initial denaturation at 94°C for 2 min, followed by 30 cycles (94°C for 30 s, 44°C for 45 s, 72°C for 90 s) and finally one cycle at 72°C for 20 min. The PCR product was separated against 100 bp + 1.5 Kb + 3 Kb DNA Ladder (from SibEnzyme Ltd, Russia) on 1.5% agarose gel. The ISSR bands were detected on UV-transilluminator and photographed by Gel documentation system.

RESULTS

Morphological characters affected by water stress

Drought treatment caused reduction in leaf number and area in all cultivars under study as compared to the normally irrigated genotypes (data are not given, but this was just noted). Plant height and tiller number also decreased under drought conditions. Five genotypes out of the 9 barley cultivars could withstand water stress and could set normal seed under water stress conditions. These cultivars are ALEXES, Giza 121, Giza 122, Giza 123 and Giza 128, whereas Jazan, Qassim, Giza 123 and Giza 2000 could not resist water stress condition and failed to complete their life cycle and produced proper seeds.

LEA gene amplification

A 676 bp band resulted from the amplification of *LEA* gene using its specific primers as seen in Figure 1. The *LEA* gene amplicon (676 bp) appeared in five (ALEXES, Giza 121, Giza 122, Giza 123 and Giza 128) barley genotypes and absent in Jazan, Qassim, Giza 123 and Giza 2000.

Table 1. SSR primers name, nucleotide sequence, chromosomal location and annealing temperature.

S/N	Primer name	Sequence	Chromosomal location	Annealing temperature (Ta °C)
1	HVB23D F: HVB23D R:	5'-ggTAgCAgACCgATggATgT-3' 5'-ACTCTgACACgCACgAACAC-3'	4 (4H)	55
2	MGB396 F: MGB396 R:	5'-CgCTAgCTTgTTTCTCgTTTg-3' 5'-TCgCATggCATCAACTACAg-3'	4 (4H)	54
3	MGB402 F: MGB402 R:	5'-CAAgCAAgCAAgCAgAgAgA-3' 5'-AACTTgTggCTCTgCgACTC-3'	5 (1H)	53
4	HVGLUEND F: HVGLUEND R:	5'-TTCgCCTCCATCCCACAAAg-3' 5'-gCAgAACgAAAgCgACATgC-3'	5 (1H)	55
5	MGB371 F MGB371 R:	5'-CACCAAgTTCACCTCgTCCT-3' 5'-TTATTCAggCAGCACCATTg-3'	6 (6H)	53
6	MGB356 F: MGB356 R:	5'-TggTCTggAgCTCTCAACAg-3' 5'-AAgCCACATTgAAggAgCAC-3'	6 (6H)	54
7	EBmac624 F: EBmac624 R:	5'- AAAAgCATTCAACTTCATAAgA-3' 5'- CAACgCCATCACgTAATA-3'	6 (6H)	48
8	Bmag210 F: Bmag210 R:	5'-ACCTACAgTTCAATAgCTAgTACC-3' 5'-gCACAAAACgATTACATCATA-3'	6 (6H)	53
9	MGB384 F: MGB384 R:	5'-CTgCTgTTgCTgTTgTCgTT-3' 5'-ACTCggggTCCTTgAgTATg-3'	7 (5H)	54
10	BMS02 F: BMS02 R:	5'-AgAgTAgTggAAAgAAAgTT-3' 5'-TggTAgTgAgATgAggTgAC-3'	7 (5H)	48
11	MGB318 F: MGB318 R:	5'-CggCTCAAggTCTCTTCTTC-3' 5'-TATCTCAgATgCCCCTTTCC-3'	7 (5H)	55

Table 2. Names and nucleotide sequences of ISSR primers used in fingerprinting the studied barley cultivars.

Primer name	Sequence
ISSR-814	$(CT)_8$ TG
ISSR-844A	$(CT)_8$ AC
ISSR-844B	$(CT)_8$ GC
ISSR-HB 8	$(GA)_6$ GG
ISSR-HB 9	$(GT)_6$ GG
ISSR-HB10	$(GA)_6$ CC
ISSR-HB11	$(GT)_6$ CC
ISSR-HB13	$(GAG)_3$ GC
ISSR-HB14	$(CTC)_3$ GC
ISSR-HB15	$(GTG)_3$GC

SSR analysis

Nineteen SSR markers were produced as a result of fingerprinting the nine barley genotypes under study using eleven SSR primers. Seven primers produced only one marker (MGB396: 200 bp, MGB402: 1000 bp, MGB371: 1400 bp, MGB356: 1300 bp, Bmag210: 200 bp, MGB384: 250 bp and BMS02: 1450 bp), two primers produced two markers (HVGLUEND: 280 and 320 bp and MGB318: 400 and 600 bp) and two primers produced four markers (HVB23D: 500, 1100, 1200 and 1300 bp and EBmac624: 300, 350, 400 and 1900 bp). Figure 2 shows the amplified SSR markers in the nine genotypes under study using primer combination MGB371, whereas Table 3 illustrates the amplicons that resulted from fingerprinting the studied genotypes using the 11 primer combinations.

ISSR analysis

Thirty-nine ISSR markers were produced as a result of fingerprinting the barley genotypes under study using ten

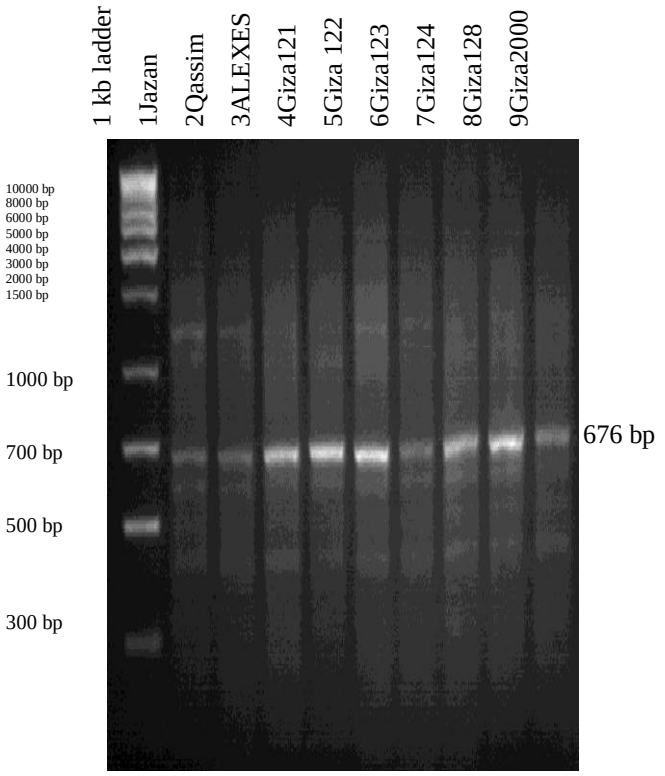

Figure 1. Amplified *LEA* gene in the studied genotypes using *LEA* gene specific primer.

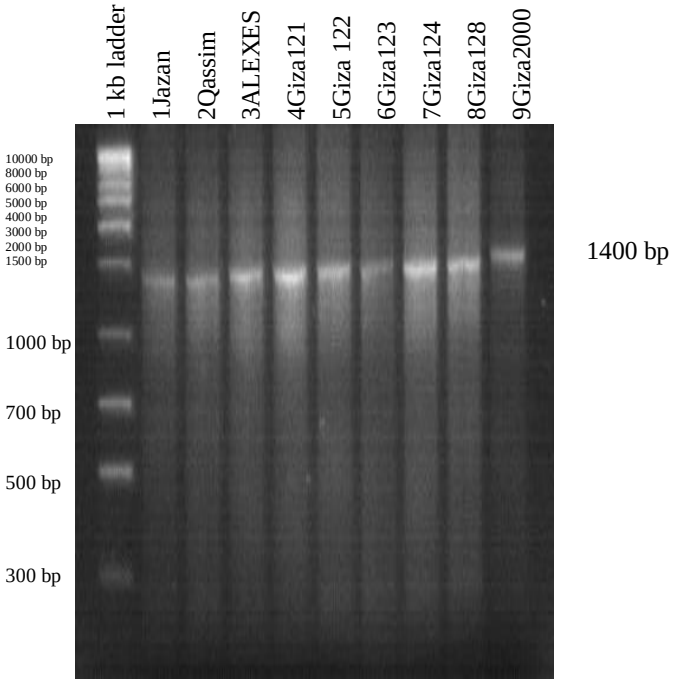

Figure 2. SSR marker amplification in the nine barley genotypes under study using primer combination MGB371.

ISSR primers. Figure 3 shows examples of amplified ISSR markers using HB14 ISSR primer. Number of ISSR markers per primer and their molecular weight range are given in Table 4.

Four markers (250, 400, 450 and 500 bp) were obtained from using primer 814, six ISSR markers (450, 500, 530, 700, 730 and 1000 bp) were obtained from using primer 844 A, three markers (400, 520 and 540 bp) were obtained from using primer 844B, three markers (530, 730 and 800 bp) were obtained from using primer HB 8, five markers (320, 400, 450, 700 and 800 bp) were obtained from using primer HB 9, three markers (480, 600 and 650 bp) were obtained from using primer HB 10, two marker (380 and 500 bp) were obtained using primer HB 11, three markers (300, 500 and 800 bp) were obtained from using primer HB 13, six markers (240, 430, 500, 600, 620 and 1000 bp) were obtained using primer HB 14 and four markers (190, 200, 700 and 800 bp) were obtained from using primer HB 15.

The phylogenetic relationships between the studied barley genotypes based on SSR and ISSR data analysis

All SSR and ISSR data were analyzed using NTSYS-PC2 program for addressing the genetic relationship among the studied genotypes as shown in Figure 4.

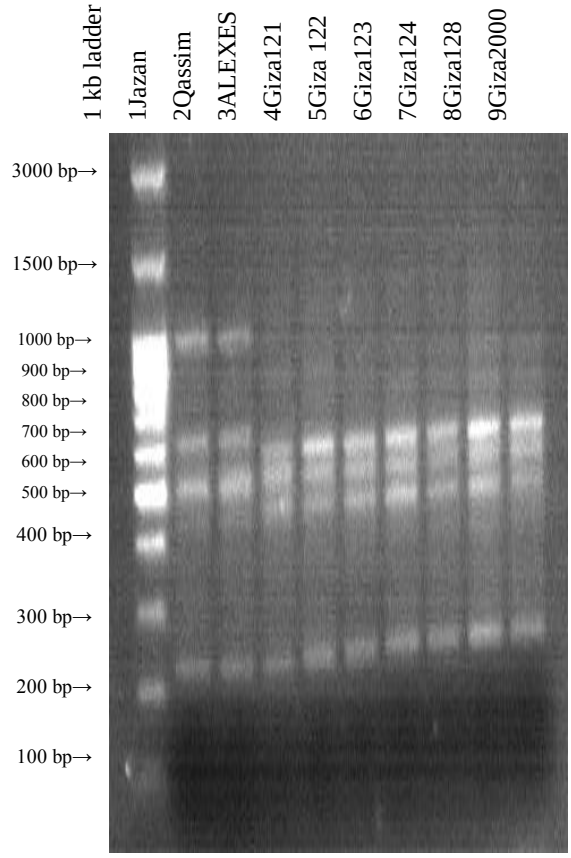

Figure 3. ISSR markers of the studied barley genotypes using HB14 ISSR primer.

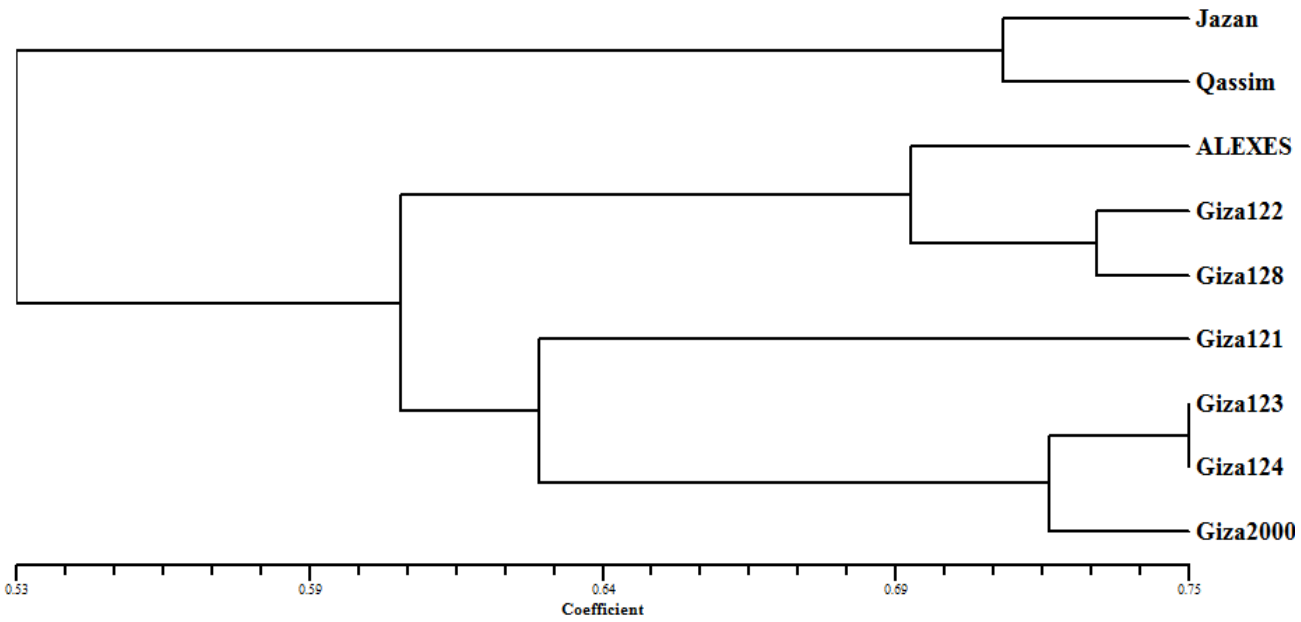

Figure 4. The phylogenetic relationships of the studied genotypes based on SSR and ISSR data using NTSYS-pc program.

Table 3. Number of SSR markers resulting per primer and their molecular weight range in the nine barley genotypes under study.

S/N	Primer name	Number of markers produced	Molecular Weight range (bp)
1	HVB23D	4	500 to 1300
2	MGB396	1	200
3	MGB402	1	1000
4	HVGLUEND	2	280, 320
5	MGB371	1	1400
6	MGB356	1	300
7	EBmac624	4	300 to 1900
8	Bmag210	1	200
9	MGB384	1	250
10	BMS02	1	1450
11	MGB318	2	400, 600

Table 4. Number of ISSR markers produced per primer and their molecular weight range.

Primer name	Number of markers resulted	molecular weight range bp
ISSR-814	4	250-500
ISSR-844A	6	450-1000
ISSR-844B	3	400-540
ISSR-HB 8	3	530-800
ISSR-HB 9	5	320-800
ISSR-HB10	3	480-650
ISSR-HB11	2	380-500
ISSR-HB13	3	300-800
ISSR-HB14	6	240-1000
ISSR-HB15	4	190-800
Total	39	

In this study, barley genotypes were divided in two clusters. The first cluster consisted of Jazan and Gassim (drought sensitive cultivars) and the other cluster is divided into two subclusters. The first subcluster contains ALEXES, Giza122 and Giza 128. The latter two are more related to each other than to ALEXES and all are drought tolerant genotypes. The other subcluster consists of two sub-sub clusters. The first one contains Giza 121 (drought tolerant genotype), whereas the other one contains Giza 123, Giza 2000 (drought sensitive) and Giza 124 (drought tolerant). Giza 123 and Giza 124 are closely related to each other than Giza 2000.

DISCUSSION

Water stress limits plant growth and the productivity of many crops (Lopes et al., 2004). In the current study, drought treatment caused reduced leaf area in all cultivars under study as compared to the normally irrigated genotypes. It was found that the advantage of cultivars with a slower growth in harsh environments is related to low demands of water and therefore will not exhaust the limited soil water reserve (Poorter, 1989). Subsequently, the decrease in leaf number can be of great interest in reducing water losses under conditions of lack of water. In the present work, plant height and tiller number decreased under drought conditions. This result is consistent with the study of Ivandic et al. (2000), who noted the same phenomenon. Also, leaf area (LA) reduced significantly within genotypes under water stress conditions.

Drought treatment affected leaf production since appearance of green leaves (GLN) was reduced in all cultivars at the end of the drought period. Barley is one of the main temperate cereals that best adapts to water shortage (Sánchez-Díaz et al., 2002). Specific leaf area (LA) differed significantly between genotypes under water stress conditions. The present results showed that the studied genotypes revealed different drought tolerance abilities; ALEXES, Giza 121, Giza 122, Giza 123 and Giza 128 genotypes were drought tolerant, whereas Jazan, Qassim, Giza 123 and Giza 2000 were sensitive to drought. Variation in drought tolerance could be a part of the drought resistance mechanisms developed by barley and could be exploited in breeding programs for improving water stress tolerance. The result of *LEA* proteins gene is consistent with drought tolerant of the studied genotypes (ALEXES, Giza 121, Giza 122, Giza 123 and Giza 128). This emphasizes that this gene confers drought tolerance in barley and other higher plants (Wang et al., 2006).

In the present study, SSR and ISSR were used for fingerprinting the barley genotypes under study so as to compare between other molecular markers conferring drought tolerance, that is, *LEA* gene and the actual drought tolerance capacity. SSR and ISSR data analysis using NTSYS-pc divided the studied genotypes under study into two clusters. The drought sensitive Jazan and Gassim genotypes were clustered alone in one group distant from all other genotypes, whereas the other sensitive cultivars (Giza 123 and Giza 2000) were clustered together with Giza 124 (drought tolerant). The other drought tolerant cultivars (ALEXES, Giza 121, Giza 122 and Giza 128) were clustered together in the other group. Similarly, a correlation between proline and abscissic acid accumulation and abiotic stress is not always so apparent and is not correlated with salt tolerance in barley (Chen et al., 2007; Widodo et al., 2009; Thameur et al., 2011). However, increasing amounts of data suggest that proline has certainly regulatory functions, controls plant development and acts as a signaling molecule (Szabados and Savouré, 2010).

Molecular makers such as restriction fragment length polymorphism (RFLP), random amplified polymorphic DNAs (RAPDs), amplified fragment length polymorphisms (AFLPs) and single nucleotide polymorphisms (SNPs) can provide a measure of genetic differences dispersed upon the genome and help as markers correlated to different traits like drought tolerance (Teulat et al., 1998). These markers have been traditionally used by plant geneticists and breeders to study intraspecific genetic variability (Heun et al., 1997; Badr et al., 2000; El Rabey et al., 2002; El Rabey and Al-Malki, 2011). These markers are used efficiently in gene banks for making genetic fingerprint of the local, wild, landraces and cultivated genotypes in order to keep the right of each country in its local resources according to the new laws of intellectual property.

The drought tolerance gene (*LEA*) or markers found in this study should provide useful information for understanding how different barley genotypes respond to drought stress at the reproductive stage and how drought-tolerant genotypes can adapt to drought-stress conditions. This will facilitate understanding of the molecular mechanisms of drought tolerance, and also facilitate the genetic improvement of barley through marker-assisted selection or gene transformation.

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