Research Article
Development of Agronomical and Molecular Genetic Markers Associated with Salt Stress Tolerance in Some Barley Genotypes

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Abstract: This study aimed to develop molecular marker (s) associated with salt tolerance in barley using Inter Simple Sequence Repeat (ISSR) and Simple Sequence Repeat (SSR) markers and usefulness of both markers, to detect possible specific markers to be utilized in the barley future breeding programs for salt tolerance. From filed evaluation, genotypes Giza 123, Rehan-03 and Saiko were found to be salt tolerant while Beecher, Line 1 and line 2 were sensitive. Using 10 ISSR primers low resolution to distinguish the two barley groups was found. However, SSR primer HVM09 exhibited a band with molecular size of 125 bp which could be considered a positive molecular marker associated with salt tolerance. Based on phylogenic tree using rooted Neighbor Joining (NJ) the dendrograms constructed with SSR marker revealed two main genetic clusters, the first include the all tolerant genotypes in and were found in closely related; since, Giza 123 have similarity 89% with Rehan-03. On the other hand, second cluster contains the sensitive genotypes, similarity value 84% was recorded between line 1 and line 2 which indicated that these three genotypes were closely related to each other and this is reflected from their response to salt stress.

Keywords: *Hordeum vulgare*, ISSR, molecular markers, salt stress, SSR

INTRODUCTION

Barley (*Hordeum vulgare* L.) is the fourth most cultivated crop of the world. Environmental stresses such as low water availability, salinity and mineral toxicity frequently affect plants in agricultural systems and represent major limitations to the yield and quality of barley and other crops. Salinity is a major abiotic stress affecting crops in Egypt and throughout the world. More than 800 million hectares of land are salt affected globally, accounting for more than 6% of the total land area (Munns and Tester, 2008). Egypt is one of the countries that suffer severe salinity problems; over 33% of the cultivated land which comprises only 3% of total land area in Egypt is already saline (Ghassemi *et al*., 1995). Moreover, the Egyptian Government has spent large sums on reclamation, to solve salinity problems in irrigated area. Saline irrigation affects on germination and growth parameters of barley and decreased with increasing in salinity level (Naseer *et al*., 2001; Taghipour and Salehi, 2008). Moreover, most of studies on barley germpalsm have long been based on morphological, physiological and biochemical (Massood *et al*., 2003; Liu *et al*., 2000).

Several biochemical techniques have been used to complement morphological examination of barley, Canci *et al*. (2003). Yet, characterization with these kinds of markers was not very efficient for barley varieties due to the low levels of allelic variation, the high degree of genetic relationship among the different varieties. Molecular markers have been proved to be valuable tools in the characterization and evaluation of genetic diversity within and between species and populations and to find marker related to specific trait (s). It has been showed that different markers might reveal different classes of variation (Powell *et al*., 1996; Russell *et al*., 1997). It is correlated with the genome fraction surveyed by each kind of marker, their distribution throughout the genome and the extent of the DNA target which is analyzed by each specific assay (D´avila *et al*., 1999).

Furthermore, DNA techniques allow the assessment of a theoretically unlimited number of polymorphic marker loci (Nguyen *et al*., 2004). Varieties of molecular markers were used to evaluate the extent of genetic variability. Among these markers Simple Sequence Repeat or microsatellite (SSR) is the marker of choice for many genetic analyses in barley.

SSR markers have a number of advantages, such as the high level of polymorphisms, locus specificity, co-dominance, reproducibility, convenience through using PCR and random distribution throughout the genome (Powell *et al.*, 1996). It is ideal for marker assisted breeding (Deric *et al.*, 2005), genetic mapping (Ramsay *et al.*, 2000). Finally SSR marker is technically efficient, cost-effective to use and are available for barley (Ramsay *et al.*, 2000; Saghai-Maroof *et al.*, 1994). Inter simple sequence repeat (ISSR) marker, which involve PCR amplifications of DNA using a primer, composed of a microsatellite sequence by 2-4 arbitrary, could be used to assess diversity and genetic marker (Qian *et al.*, 2001). It has been used for cultivar identification for wheat (Nagaoka and Ogihara, 1997) and barley (Tanyolac, 2003; Fern´andez *et al.*, 2002; Brantestem *et al.*, 2004). Thus, development molecular genetic markers associated with salt stress tolerance in barley genotypes has become a crucial task in dealing with salinity problems in Egypt.

Until now, very few publications have been analyzed the pattern of genetic diversity by ISSR or SSR markers within Egyptian barley genotypes. However, there is no any published data for those markers and salt tolerance in barley genotypes grown in Egypt (Mariey, 2013). Ramsay *et al.* (2000) developed a new SSR based markers for 16 barley genotypes. At same time, Ellis *et al.* (2002) tested the variation in SSR on two loci and found that the SSR has a wide range of alleles and provides an extreme example of the effects of barley domestication. Moreover, barley traits related to salt tolerance were mapped using SSR (Ellis *et al.*, 2002; Eleuch *et al.*, 2008). Recently, high level of allelic diversity among barley landraces were reported by Naeem *et al.* (2011) they evaluated genetic diversity among landraces of barley from different geographical regions using SSR markers. The present study aimed to develop marker (s) associated with salt tolerance in barley using ISSR and SSR markers and usefulness of both markers, to detect possible specific markers to be utilized in the future breeding for salt tolerance in barley.

MATERIAL AND METHODS

Barley genotypes: Six barley (*H. vulgare* L.) genotypes were selected from 20 genotypes based on their tolerance/sensitivity to salinity stress (Table 1). Barley genotypes were kindly provided by Sakha Barley Research Department, Field Crops Research Institute, Agricultural Research Center and Giza, Egypt. The selection criteria of the six genotypes were based their salt stress tolerance and the yield performance.

Field experiments: Two experiments were carried out in this study, the first experiment was conducted during 2009/2010 in EL-Serw (as a saline soil; EC = 11.6). using twenty genotypes from the pure genetic stock of Barley Research Department used as a preliminary evaluation experiment (data not shown). The selected

Table 1: Name pedigree and degree of salt tolerance of six barely genotypes

No	Name	Pedigree	Degree of tolerance
1	Giza 123	Giza 117 /FAO86	Tolerance
2	Rihane-03	As46//Avt/Aths	Tolerance
3	Saiko	---	Tolerance
4	Beecher	Introduced to Egypt and named Giza 118	Sensitive
5	Line 1	ICB890775-7AP-0AP-0AP-10AP-0AP-1AP-0AP	Sensitive
6	Line 2	ICB95-0281-0AP-6AP-0AP-7TR-1TR-0AP	Sensitive

six genotypes were grown in the field in two locations (Sakha control; EC = 4.5 and El-Serw saline soil; EC = 12.8) in the second 2010/2011. The six genotypes were planted in a Randomized Complete Block Design (RCBD) with three replicates each plot consisted of a genotype, which was planted in plots of four rows 2.0-m long and 20-cm apart (plot area = 1.6 m^2) with three replications.

Studied traits: Nine growth parameters for the six studied barley genotypes were taken on 10 individual plants which had been randomly taken from the central rows of each plot are seedling rate (%), days to 50% heading (days) (DH), days to maturity (days), plant height (cm), number of tillers/m^2, number of spikes/m^2, number of grains/spike^1, 1000-grains weight (g) and grain yield (kg/m^2) These traits are most likely affected by salt stress and soil conditions.

Molecular analysis: DNA was isolated by CTAB method (Doyle and Doyle, 1990). Ten SSR and 10 ISSR primers were used in this study, sequences of the used primers illustrated in Table 2. PCR amplification was performed in a total volume 20 µL containing 2.5 µL 10 X buffer, 1 µL 25 mM MgCl2, 2 µL 2.5 mM dNTPs, 2 µL 10 pmol primer, 1 µL 50 ng of genomic DNA and 0.16 µL Taq DNA polymerase (5 units/µL). PCR amplification was for one cycle at 95°C for 5 min. Then 35 cycles were performed as follows: 1 min at 95°C for denaturation, 30 s at (38°C for ISSR and from 55°C to 58°C for SSR) and 45 s at 72°C for extension. Reaction was incubated at 72°C for 7 min then at 4°C. The products were separated by electrophoresis using 2% agarose gel in 0.5 x TBE buffer against 100 bp DNA Ladder. Bands were detected with Ethidium Bromide staining and visualized under UV light, then photographed on Gel Documentation.

Data analysis: Data were statistically analyzed as a Randomized Complete Block Design (RCBD) using analysis of variance (ANOVA) for each season and over all the two seasons as a combined analysis using COSTAT software. Phylogenetic trees were constructed based on similarity matrix obtained with Neighbor Joining (NJ) method using Jaccard formula djk = M/(M+N). The relationships between genotypes were displayed as dendrogram using the NTSYSpc 2.01 software package (Rohlf, 1998).

RESULTS AND DISCUSSION

Field screening: Mean performances of six barely genotypes under control and salt stress as well as the combined analysis for all studied traits are presented in Table 3. Two genotypes (Giza 123 and Rehan-03) were showed the highest mean performance values for most studied traits under both locations (EL-Serw and Sahka)

Table 2: Primer sequences of SSR used in PCR analysis

	SSR primers			ISSR primers	
No	Primer name	Sequence 5' 3'	No.,	Primer name	**Sequence 5' 3'**
1	Bmag 06	F: TTAAACCCCCCCCCTCTAG R: TGCAGTTACTATCGCTGATTTAGC	1	ISSR 1	(GA) 8 T
2	Bmac 415	F-GAAACCCATCATAGCAGC R-AAACAGCAGCAAGAGGAG	2	ISSR2	(CA) 8 G
3	Bmag 0209	F: CTAGCAACTTCCCAACCGAC R:ATGCCTGTGTGTGGACCAT	3	ISSR3	(AG) 8G
4	Bmac 0316	F: ATGGTAGAGGTCCCAACTG R: ATCACTGCTGTGCCTAGC	4	ISSR4	(AC) 8GA
5	Bmag 0770	F: AAGCTCTTTCTTGTATTCGTG R: GTCCATACTCTTTAACATCCG	5	ISSR5	(CT) 8TT
6	Xgwm 153	F: GATCTCGTCACCCGGAATTC R: TGGTAGAGAAGGACGGAGAG	6	ISSR6	(AC) 8CA
7	Xgwm268	F: AGGGGATATGTTGTCACTCCA R: TTATGTGATTGCGTACGTACCC	7	ISSR7	(GA) 8GG
8	HvAMY2	F:CTGTAAGTGAGACAATCGACA R:CAGTTGAACCCCTGAAAG	8	ISSR8	(GA) 8C
9	HVM09	F: CTTCGACACCATCACCCAG R: ACCAAAATCGCATCGAACAT	9	ISSR9	(GATA) 2 (GACA) 2
10	HVLEU	F: TTGGAAGTGTACAGCAATGGAG R: TGAAAGGCCCCACAAGATAG	10	ISSR10	GGGGT)3G

Table 3: Overall mean values of nine studies characters for six genotypes and their interaction under EI-Serw and Sakha location

|	Seedling rate %			Days to heading (days)			Daye to maturity (days)		
Genotypes	Serw	Sakha	Combined	Serw	Sakha	Combined	Serw	Sakha	Combined
Giza 123	78.3	100.0	89.2	91.3	101.3	96.3	136.0	141.0	138.5
Rihaen 03	73.3	100.0	86.7	97.3	99.0	98.2	138.3	145.0	141.7
Saiko	65.0	100.0	82.5	87.7	96.3	92.0	130.7	136.0	133.3
Becheer	50.0	80.0	65.0	97.0	102.3	99.7	140.7	147.0	143.8
Line 1	70.0	83.3	76.7	95.3	103.7	99.5	139.0	144.7	141.8
Line 2	56.7	86.7	71.7	97.3	101.3	99.3	139.3	142.7	141.0
Average	65.6	91.6	78.6	94.3	100.6	97.5	137.3	142.7	140.0
F.Test	***	**	Ns	*	*	Ns	***	***	Ns
LSD 0.05	9.92	8.78	7.86	5.64	4.12	3.16	2.39	3.45	1.95
C.V%	8.32	5.26	8.35	3.29	2.24	2.71	0.96	1.32	1.17
	Plant height (cm)			**Number of tillers/m²**			**Number of spikes/m²**		
Genotypes	Serw	Sakha	Combined	Serw	Sakha	Combined	Serw	Sakha	Combined
Giza 123	80.7	124.3	102.5	466.7	479.3	473.0	403.3	443.3	423.3
Rihaen 03	79.7	112.0	95.8	366.7	423.3	395.0	296.7	384.0	340.3
Saiko	82.3	116.7	99.5	296.7	450.7	373.7	173.3	450.7	312.0
Becheer	68.7	115.7	92.2	243.3	384.0	313.7	200.0	298.0	249.0
Line 1	81.3	107.7	94.5	343.3	400.7	372.0	223.3	378.7	301.0
Line 2	76.3	113.0	94.7	298.7	353.3	326.0	303.3	298.7	301.0
Average	78.2	114.9	96.5	335.9	415.2	375.6	266.7	375.6	321.1
F.Test	***	Ns	**	***	*	Ns	***	***	Ns
LSD 0.05	3.41	10.52	5.01	73.21	65.97	53.66	53.94	31.21	70.82
C.V%	2.39	5.03	4.33	11.98	8.73	11.93	11.09	4.56	18.4
	Number of grain/spike			**1000 grain weight (g)**			**Grain yield (Kg/m²)**		
Genotypes	Serw	Sakha	Combined	Serw	Sakha	Combined	Serw	Sakha	Combined
Giza 123	62.0	66.0	64.0	51.2	55.0	53.1	1.0	1.2	1.1
Rihaen 03	58.0	58.0	58.0	48.2	49.2	48.7	1.0	1.2	1.1
Saiko	64.0	70.0	67.0	50.2	58.2	54.2	0.4	1.1	0.7
Becheer	46.0	48.0	47.0	44.3	46.6	45.4	0.4	1.0	0.7
Line 1	52.0	54.0	53.0	41.5	44.4	42.9	0.6	1.1	0.8
Line2	50.0	54.0	52.0	43.1	49.8	46.5	0.3	1.2	0.8
Average	55.3	58.3	56.8	46.4	50.5	48.5	0.6	1.1	0.9
F.Test	***	*	Ns	*	**	Ns	***	Ns	***
LSD 0.05	9.49	12.23	6.95	3.22	5.88	4.21	0.07	0.23	0.14
C.V%	9.34	11.52	10.22	3.81	6.40	7.24	6.95	11.26	11.10
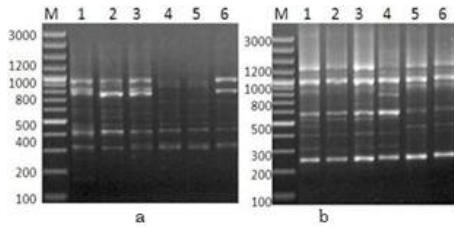
Fig. 1: PCR amplification profile generated from genomic DNA of six barley genotypes with ISSR3 (a) and ISSR6 (b)
M-marker = 100bp 1-Giza 123, 2-rehan-03, 3-Saiko, 4-Beecher, 5- Line 1 and 6- Line 2
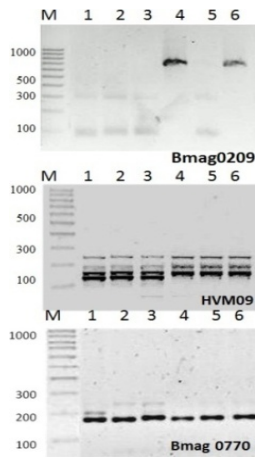

Fig. 2: PCR amplification profile generated from genomic DNA of six barley genotypes with SSR primers, Bmag0209, HVM09 and Bmag 0770. M-marker = 100bp 1-Giza 123, 2-rehan-03, 3-Saiko, 4-Beecher, 5-Line 1 and 6- Line 2
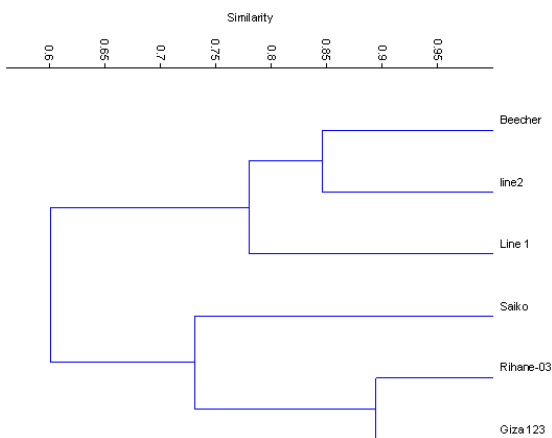

Fig. 3: Dendrogram showing grouping of six barley genotypes based on jaccard derived from SSR markers using UPGMA analysis

and in their combined, On the other hand, Becheer and line 2 showed the lowest mean performance values for most studied traits under both locations and their combined.

Regarding the mean squares for all genotypes were significant and high significant for all traits under salt stress, control and their combined data but the mean squares were non-significant for plant height and grain yield under Sakha only. These results are in a good agreement with Mariey (2004) and Oraby *et al*. (2005) they found that the salinity reduced number of tillers, plant height, days to heading, number of spike/plant[1] and grain yield. For interaction (GxL) between Locations (L) and Genotypes (G) the combined analysis showed that there was non-significant effect of all traits expect plant height and grain yield was significant. Different types of significant of agronomic traits have been observed in barley under salinity (Naseer *et al*., 2001; Ahmad *et al*., 2003; Mariey, 2004; Eleuch *et al*., 2008; Taghipour and Salehi, 2008). Environmental factors can modify the tolerance of plant. Plant phenotype is a product of genotype and environment. Salinity and other environmental effects interact in several ways that may obscure inheritance studies of salt tolerance.

SSRs analysis: Among the 10 primers used, three showed monomorphic band profiles (Xgwm 153, Xgwm 268 and Bmag 06) and two showed no amplification bands (HVLEU and HvAMY2) thus, were discarded. The remaining five primers (Bmag 0209, Bmac 0316, Bmac 415, Bmag 770 and HVM09) generated clear pattern polymorphism (Fig. 1 and Table 4).

The five discriminatory primers were used to evaluate the genetic diversity among the studied barley genotypes. These primer pairs revealed a total of 16 alleles ranging from one to four alleles per locus (Table 4). Four polymorphic bands ranged between 125 and 250 bp generated by HVM09 primer, one of them recorded only in tolerant genotypes with size around 125 bp, which is considered as a positive marker for salt stress in barley. For all genotypes, the highest number of polymorphic bands was developed by the primer Bmac 415 showed one band with polymorphic percentage 100% but could not show any distinguish among the studied genotypes. Based on phylogenic tree using rooted Neighbor Joining (NJ) the dendrograms constructed with SSR marker revealed two main genetic clusters Fig. 3, the first cluster include the all tolerant genotypes and were found in closely related since, Giza 123 have similarity 89% with Rehan-03; which it in a good agreement with field evaluation data. On the other hand, second cluster contains the sensitive genotypes (Beecher, Line 1 and Line 2); similarity value 84% was recorded between line 1 and line 2 which indicated that these three genotypes were closely related to each other and this is reflected from their response to salt stress as showed in Table 5.

ISSR analysis: In the present study, ISSR was used to find marker related to salt stress and to assess genetic variation of barley under salt stress. This method provides an alternative choice to other system for

Table 4: ISSR and SSR primers, their amplified fragments, polymorphic and the polymorphic and the polymorphic percentage

SSR Primers	Amplified bands			ISSR primers	Amplified bands		
	Total	Polymorphic	Polymorphic %		Total	Polymorphic	Polymorphic %
HVLEU	0	0	0	(GA) 8 T	5	5	100
HVM09	4	1	25	(CA) 8 G	0	0	0
Bmag 06	1	0	0	(AG) 8G	6	4	66.6
Xgwm 153	1	0	0	(AC) 8GA	2	2	100
Xgwm 268	2	0	0	(CT) 8TT	7	5	71.4
Bmac 0316	5	4	80	(AC) 8CA	11	8	72.7
Bmac 0209	3	3	100	(GA) 8GG	4	3	75..0
Bmag 770	3	2	25	(GA) 8C	3	2	66.6
Bmac 415	1	1	100	(GATA) 2 (GACA) 2	0	0	0
HVAMY2	**0**	**0**	0	(GGGGT) 3G	3	3	100

Table 5: SSR similarity indices based on Jaccard

	Giza 123	Rihane-03	Saiko	Beecher	Line 1
Giza 123	1				
Rihane-03	0.89	1			
Saiko	0.68	0.77	1		
Beecher	0.57	0.57	0.73	1	
Line 1	0.57	0.57	0.73	0.71	1
line2	0.50	0.5	0.62	0.84	0.84

Table 6: ISSR similarity indices based on Jaccard

	Giza 123	Rihane-03	Saiko	Beecher	Line 1
Giza 123	1				
Rihane-03	0.95	1			
Saiko	0.81	0.77	1		
Beecher	0.68	0.71	0.68	1	
Line 1	0.56	0.59	0.63	0.65	1
Line2	0.59	0.62	0.59	0.60	0.72

Table 7: Combined SSR and ISSR based similarity indices based on Jaccard

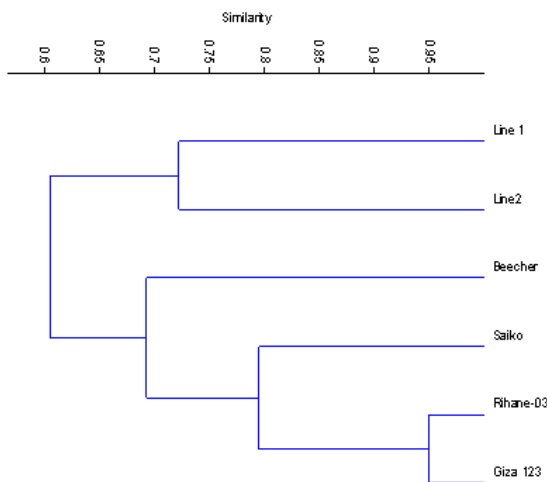|	Giza 123	Rihane-03	Saiko	Beecher	Line 1
Giza 123	1				
Rihane-03	0.91	1			
Saiko	0.73	0.77	1		
Beecher	0.61	0.62	0.71	1	
Line 1	0.57	0.58	0.69	0.68	1
line2	0.53	0.540	0.61	0.73	0.79

Fig. 4: Dendrogram showing grouping of six barley genotypes based on jaccard derived from ISSR markers using UPGMA analysis

obtaining highly reproducible marker. Polymorphism was revealed in the banding patterns in the six barley genotypes. Among the 10 ISSR primers used, five of them generated polymorphic PCR products. For a total
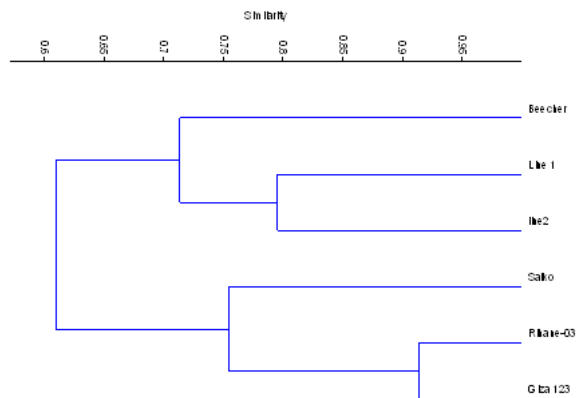

Fig. 5: Dendrogram showing grouping of six barley genotypes based on jaccard derived from SSR and ISSR markers using UPGMA analysis

41 bands were generated. Highest number of bands was generated by using primer ISSR6, ISSR5 with sequence of (AC) 8CA, (CT) 8TT respectively as shown in (Fig. 2 and Table 4). Whereas primer ISSR1 with sequence (GT) 8T was generated highest polymorphism (100%) compared to other ISSR primers, followed by primer ISSR7 with sequence (GA) 8 GG with polymorphism (75%). Dendrogram tree revealed two main genetic clusters Fig. 3. The first cluster includes the tolerant genotypes in one sub-cluster and Beecher as a sensitive genotype with similarity less than 70%. On the other hand, the second cluster contains the sensitive genotypes (Line 1 and Line 2). The highest similarity value 95% was recorded between the two genotypes Giza 123 and Rehan-03, followed by Giza 123 and Saiko with similarity 81% as shown in (Table 6). Moreover, similarity value 77% was recorded between Rehan-03 and Saiko, which indicated that these three genotypes were closely related to each other and this is reflected from their response to salt stress. On the other hand, the lowest value 56% was recorded between the two genotypes Giza 123 and line 1 indicating that these cultivars were genetically distant genotypes in their salt stress. When ISSR and SSR data were combined give similar data to SSR which the two barley groups were clustered as shown in (Fig. 4 and Table 7).

Markers validation on independent genotype of different genetic background is essential in determining the effectiveness and reliability of the markers to predict phenotypic (Cakir *et al.*, 2003). Markers should also be validated by testing for the presence of the markers on a range of cultivars and other important genotypes. Therefore, marker assisted selection for salinity tolerance could be genotype resistance specific. Based on primer HVM 09 showed the lowest polymorphism (25%) and found to be positive marker for salt stress in barley, and Bmag 0209 showed (100%) polymorphism but no clear band related to salt tolerant

Genetic relationships between barley genotypes revealed by genetic similarity at SSR levels were in agreement with their roles in agricultural production and breeding (Karakousis *et al.*, 2003) argued the usefulness of polymorphic SSR markers for the discrimination of barley. Moreover, Kanbar (2011) found same to our result when he study genetic diversity in barley using ISSR. For this study we can consider that these genotypes which showed salt tolerance could serve as potentially novel germplasm that could be exploited for the development of new breeding lines with high level of salinity tolerance and to accelerate genetic advancement in barley and cost-efficient than conventional screening under saline field conditions. Finally using SSR marker we could be found clear distinguish between the two groups and find a positive marker related to salt. However, ISSR cannot drown that clear picture, so ISSR may be useful for studying genetic diversity but not to study marker related to specific trait (s). It could be concluded that markers differ in their ability to differentiate individuals, the mechanism of detecting polymorphism, genome coverage and the ease of application. They can be complementary to each other depending on technical availability. Some of these markers can be linked to stress tolerance genes in tolerant genotypes that can be transferred to good yield but sensitive cultivar (s) through Marker-Assisted Selection (MAS) (Zhong *et al.*, 2006; Miedaner and Korzun, 2012). There are some efforts towards breeding salinity tolerance in plant via MAS (Thomson *et al.*, 2010; Ashraf *et al.*, 2012) that can be duplicated for the development of salt tolerant cultivars of barley.

CONCLUSION

SSR marker was found to drown clear distinguish among studied genotypes and find a positive marker related to salt tolerance. However, ISSR marker cannot drown that clear picture. Moreover, SSR primer HVM09 exhibited a band with molecular size of 125 bp which could be considered a positive molecular marker associated with salt tolerance. As a conclusion the use of Markers-Assisted Selection (MAS) was proved to be good alternative to the agronomic selection, that need

time, effort and labor, where it provides the breeder with the genetic markers for certain economic traits, such salt.

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