

Genetic Analysis of Salinity Tolerance in Some Barely Cultivars

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Abstract: Environmental stress, especially saline soils and saline water, are one of the most important limited factors for agricultural crops in particular all around the world. Hence, yield enhancement in agricultural crops such as barley under saline conditions is a major goal of enhancement barley salt tolerance. In the present investigation we focus to study the salinity stress tolerant among four genotypes of Egyptian barley. Leaf samples of old seedlings were collected after 30 days of grown under treatments (control, 6000) of NaCl, to evaluate the ability of the initial material to salinity tolerances. The barley genotypes differ genetically in their salt tolerance potentiality and classified to salinity stress tolerant (Giza 123, Giza124, Giza125 and Giza 126). Some physiological measurements as abscisic acid, Proline and chlorophyll content were observed under salt stress condition. Based on SSR detection related to salt tolerance association, Six SSR primers (Bmac0209, Bmac 0316, Scssr 03907, Bmag770, HVM67 and HVHOTRI) were generated clear patterns with high polymorphism and success to evaluate the association of salt tolerance detection pattern among four barley genotypes under control and salinity stress. These primers pairs revealed a total of 23 alleles ranging from three to five alleles per locus and the polymorphism information content (PIC) was enabled to measure of allelic variability and evenness at a particular locus, PIC values was ranged from 0.424 to 0.754 with primer Bmag770 and HVHOTRI, respectively.

Keywords: Barley, salt stress, physiological, molecular marker, SSR, PIC

I. Introduction

Barely, *Hordeum vulgare* L., is recognized as one of the most economic and important cereals in the world. On behalf of the area and production, barley is the fourth most important cultivated crop, following, wheat, rice and maize. It can be grown in a wide range of environmental conditions and give satisfactory yields in areas that are not suitable for growing most of the others cereals crops due to problems of abiotic and biotic stress (Mass et al., 1986; Katja et al., 2009). Abiotic stress in fact is the principal cause failure worldwide, dipping average yield for most major crops by more than 50% (Bray et al., 2000). Abiotic stress causes losses worth hundreds of million dollars each year due to reduction in crop productivity and crop failure (Shilpi et al., 2005). Along with abiotic stresses, salinity in soil and in irrigation water is very harmful and adversely affects plant growth, development and restrict yield on 40 million hectare of irrigated land in the world (Zhu et al., 2006; Yildiz et al., 2008). When salinity exceeds to optimum tolerance of a plant, the result is stress to the plant, which in turn influences its developmental, structural, physiological and biochemical processes (Jaleel et al., 2007). Moreover can cause damages to sensitive plant species by altering patterns of gene expression including change in cellular structures and impairing membrane function (Muthukumarasamy et al., 1997). For many years, breeding for salt tolerance has been an important task to increase crop productivity under salt stress and choice of parents for crossing is considered an important step in any plant breeding program aimed to an increase in the salinity tolerance of barley which could improve the profitability of some of the more than one billion salt affected hectares present in the world (El-Fadly et al., 2007). Using non-conventional approaches such as molecular marker as a strategy to obtain plants with higher performance under salt stress conditions by identify the genes and banding patterns that take place when the plant become growing under salt stress may further accelerate the progress of such breeding programs (Abd-El-Haleem et al., 2009).

Salt stress induces various biochemical and physiological responses in plants and affects almost all plant processes also induces water deficit biosynthesis by decreasing the osmotic potential and the inhibition of gibberellic acid which leads to a decreased efficiency of photosynthesis and is known to influence the chlorophyll content of plant leaves and effect on Proline (Turan et al. 2009). Therefore abscisic acid contents causes significantly increases in the endogenous content of proline amino acid and abscisic acid in comparison with that obtained from their corresponding control plants irrigated with tap water (Meloni et al 2003). Microsatellite or simple sequence repeat (SSR) markers are very useful for studying the salt stress marker and genetic diversity for several reasons, SSR markers combine a number of advantages for practical applications, as they are co-dominant and multi-allelic, stably inherited, amenable to automation and high-throughput analysis, highly variable and detect the highest level of polymorphism per locus (Roder et al., 2004). They require only small amounts of sample DNA, are easy to amplify by polymerase chain reaction (PCR), are amenable to high-throughput analysis, and are largely co-dominantly inherited, multi-allelic, highly informative, and abundant in plant genomes (Powell et al., 1996). In barley, more than 775 microsatellites have

been published (Varshney et al., 2007), and genetic maps based on microsatellites for all seven barley chromosomes are publicly available (Saghai-Marooif et al., 1994; Becker & Heun, 1995; Liu et al., 1996; Struss & Plieske, 1998; Ramsay et al., 2000; Varshney et al., 2007). Numerous studies on the analysis of genetic diversity in wild and cultivated barley have been conducted using SSRs makers (Saghai-Marooif et al., 1994; Russell et al., 2000; Struss and

Plieske, 1998; Pillen et al., 2000; Macaulay et al., 2001; Ivandic et al., 2002; Hamza et al., 2004). Marker-assisted selection (MAS) is very efficient in backcross-assisted incorporation of single recessive resistance genes (Ordon et al., 2004) as well as in pyramiding non-linked resistance genes (Werner et al., 2007). Few studies such as (Saker, 2005) have analyzed the pattern of genetic diversity via SSR markers within Egyptian barley. In the present investigation we exploration the SSR markers to investigate the salt stress markers among four Egyptian barley genotypes for salt tolerance detection. Polymorphic information content (PIC) for SSRs is affective tool to measure of a marker's informativeness, different PIC values were obtained from marker studies using different genetic materials in barley. (Khodayari et al. 2012) reported PIC values ranging from 0.01 to 0.92, the number of alleles per locus is a significant indicator of genetic diversity (Tomka et al. 2013), they have identified a total of 55 alleles at 10 microsatellite loci, and in the individual loci they have detected from 3 to 9 alleles with an average of 5.5 alleles per locus on his study on 30 barley genotypes.

II. Material and Methods

Plant Material

Four Egyptian barley landraces (*Hordeum vulgare L.*) (Giza 123, Giza 124, Giza 125 and Giza 126) were used in this investigation. Barley landraces were obtained from Field Crops Research Institute, Agricultural Research Center (ARC), Giza, Egypt. As shown in (Table 1).

Salinity experiment

Seeds of the four genotypes were sown, in plastic pots (300 mm) filled with 2 Kg of soil mixture containing clay soil, sand and petmous at 1:1:1 ratio, in the green house. 10 seeds of each were sown in each pot per entry with two replications and all pots were irrigated with tap water (300 ppm salt) up to 14 days after sowing. On day 15 salt treatments of 6000 ppm NaCl with unsalted treatments as control were applied and leaf samples from each entry were collected, after 30 days old seedlings grown under control and saline conditions and placed directly in deep freezer at -80°C until they were used for biochemical and molecular analysis.

Physiological Analysis

Abscisic acid analysis: Abscisic acid was extracted, methylated and estimated according to the method adopted by Wasfy et al. (1975).

Proline content: in the plant parts was estimated according to the method of (Bates et al., 1973), where proline estimation was done based on the following Equation:

$$\text{mmoles per gram tissue} = \frac{\frac{\text{mg proline} \times \text{ml toluene}}{\text{ml}}}{115.5} \times \frac{5}{\text{g sample}}$$

where 115.5 is the molecular weight of proline.

Quantitative Estimation of Chlorophyll

Chlorophyll was calculate according to (Arnon, 1949), where the chlorophyll was expressed as mg/g fresh tissue (Arnon, 1949). While Total Chlorophyll = [20.2 (A645) + 8.2 (A663) V/1000 x W]. Where, A663 - Absorbance at 663 nm, A645 - Absorbance at 645 nm, V - Volume of extract, W - Weight of tissue.

Molecular analysis

DNA extraction

Genomic DNA was isolated from the leaves collected after a month of sowing (eight barley samples, four plants from control and four from saline stress) using the Gen-Elute Plant Genomic DNA Miniprep Kit.

SSR primers associated to salt stress tolerance

Microsatellite Markers, DNA Extraction and PCR Amplification

Six microsatellite primers were developed on the basis of the salt-tolerant and associated to salinity stress expression from the published sequences of (Saghai-Marooif et al., 1994; Pillen et al., 2000; Ramsay et al., 2000; Karakousis, 2002) have been used for this study. The length average were ranged from 18-24 bp. Primers' sequences, chromosomal location, size range, marker type and the reference are listed in Table (2). Genotyped markers were assigned using the Grain Genes data base (<http://grain.jouy.inra.fr/cgi-bin/graingenes/browse.cgi>) (Kleinhofs & Graner, 2001).

PCR amplification and electrophoresis

PCR amplification was performed in a volume of 25 µl containing approximately 30 ng of template DNA, 1 µl of each forward and reverse primer, suitable quantity of dNTPs, MgCl₂ and Taq DNA Polymerase and PCR buffer. Reactions were conducted in Eppendorf PCR system (Germany) with initial denaturation step for 5 min at 94°C followed by 35 cycles of 94°C for 1 min, 54-56°C for 1 min and 72°C for 2 min; followed by

a final extension at 72°C for 5 min. The PCR reaction products were evaluated for polymorphisms on 3% agarose gel. After staining with 8 µl Nancy (revelation dye) for 60 min, the gels were photographed by gel documentation system.

Data scoring and statistical analysis:

To ensure the absence of artifacts, bands were carefully selected from replicated amplifications (three times). Amplified bands designated by their primer code and their size in base pairs. Data recorded as discrete variables: 1 for the presence and 0 for the absence of a similar band. Only intense and reproducible bands appearing on the gel were scored. Band scoring was analyzed using Gene Tools-gel analysis software of SPSS ver. 16. The Polymorphic Information Content value (PIC) refers to the value of a marker for detecting polymorphism within a population and depends on the number of detectable alleles and the distribution of their frequency. PIC was calculated using the equation:

$$PIC_i = 1 - \sum_{j=1}^n P_{ij}^2$$

where, PIC_i is the polymorphic information content of a marker i; P_{ij} is the frequency of the jth pattern for marker i and the summation extends over n patterns

III. Results and Discussion

Physiological Analysis

The results of abscisic acid contents in the four cultivars of barley are shown in Table (3). It is clear from the data presented that irrigated barley plants with solutions of NaCl up to 6000 ppm caused an increase in the endogenous amounts of ABA in comparison with the amounts obtained from the plants irrigated with tap water, the results indicated that Abscisic acid content was generally increased in the four cultivars under salt stress as compared to its content in plants grown under control condition of non salt stress, the increasing folds in abscisic acid content under salt treatment varied among the cultivars, it was about 167.65 to 59.60 folds in the tolerant cultivars under salt stress and about 49.16 to 33.12 under control of non salt stress. Effect of salinity stress levels on proline shows that irrigation of barley plants with salinity 6000 ppm caused significantly increases in the endogenous content of amino acid "proline" in compare to that obtained from their corresponding control (plants irrigated with tap water), generally also increased in the four cultivars under salt stress as compared to its content in plants grown under control condition of non salt stress as shown in Table (4). The increasing in proline content under salt treatment varied within the cultivars, it was about 112.17 to 119.45 under salt stress and about 15.54 to 13.12 under non salt stress, these results indicated that under salinity stress plants have mechanisms against with that which accumulation of solution components such as proline one of the primary responses of plant proportion to salinity. (Yazici et al in 2007) reported that with increasing of salinity imposed, free proline content in leaves was increased, which confirm this result also (*Hordeum vulgare* L.) he was also observed that proline as a reducer component of osmosis pressure in response to increase of salinity (Ueda et al, 2007).

On the other hand photosynthetic pigments content were affected also by salt stress and measured through analysis of Chlorophyll a and Chlorophyll B as shown in Table (5), it was observed generally decreasing of Chlorophyll A and Chlorophyll B in the four cultivars under salt stress as compared to its content in plants grown under non salt stress condition, the decreasing average of Chlorophyll A content was from 1.55 under non salt stress to 0.87 under salt treatment varied among the cultivars and the decreasing average of Chlorophyll b content was from 0.83 under non salt stress to 0.65 under salt treatment varied among the cultivars it is agreed with (Doganlar et al, 2010), he was observed that salinity has toxic effects on plants and causes of changes in metabolic activity such as reduced activity of chloroplasts, photosynthetic pigments, the rate of photosynthesis and increase of respiration rate which ultimately leads to increased production of reactive oxygen species in plant will be changing of leaf chlorophyll content by salinity stress.

Molecular Analysis

SSR associated to salt stress tolerance

Six SSR primer primers (Bmac0209, Bmac 0316, Scssr 03907, Bmag770, HVM67 and HVHOTR1) generated clear patterns with high polymorphism. (Table 6 and Figure 1). The six discriminatory primers pairs were succeeded to evaluate the genetic diversity and association of salt tolerance in eight barley samples (four under control and four under salinity stress), these primers pairs revealed a total of 23 alleles ranging from three to five alleles per locus (Table 6). For all tested genotypes, the highest number of bands was developed by the primer scssr0397 (five bands), followed by Bmac0209 and HVHOTR1 (four bands). Moreover, the primer Bmac0209 showed unambiguous bands with the eight barley genotypes with varying responses to salinity stress, it showed four bands with 100% polymorphism. Additionally, the primer Bmac0316 appear fewer bands number but have high polymorphic percentage, it showed three bands, with 100% polymorphism, while the

primer scssr0397 created five bands with 80% polymorphism. However the lowest number of polymorphism bands was found by the primer HVM67 and Bmag770 which appear three bands with 66% polymorphism. The polymorphism information content (PIC) was a measure of allelic variability and evenness at a particular locus. In this revised the PIC values ranged from 0.424 (Bmag770) to 0.754 (HVHOTR1) (Table 6). In previous studies, different PIC values were observed using different genetic materials in barley. In view of the results of Bolouri et al. (2011), the PIC values ranging from 0.8 to 0.88. Moreover (Sardou et al. 2011), reported that PIC values ranging from 0.29 to 0.89 with mean of 0.64. Whilst (Chaabane et al. 2009), reported that PIC values ranged from 0.28 to 0.60 with an average value of 0.50.

The six expressing SSR primers enabled us to discriminate all the genotypes for studding the genetic variability for salt tolerance among the improved varieties and lines, SSR patterns illustrated that there are bands appeared in all genotypes (common bands). However other bands were present in some genotypes and absent in the others (polymorphic). The appearance of some polymorphic bands may be indicated to the direct relationship with salt stress which reflect the genetic of gene defense to salinity stress tolerance in the four Egyptian barely cultivars, similar observations were also reported by (Lin et al., 1998), under stress in plants, since molecular bands were newly synthesized under stress, it appears to have a role in the mechanism of salt stress tolerance for example, which allows making biochemical and structural adjustments that enable the plant to cope with stress conditions. Markers validation in independent genotypes of different genetic background is essential in determining the effectiveness and reliability of the markers to predict phenotypic (Koyama et al., 2001; Collins et al., 2003; Cakir et al., 2003), which indicates that SSR marker, could be used in routine screening for marker-assisted selection (MAS). 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, indicated that the potential efficacy of highly informative SSR markers were efficient screening for brewing genotypes in barley. Genetic relationships among barley varieties revealed by genetic similarity at SSR levels were in agreement with their roles in agricultural production and breeding (Qian et al., 2011). As a good confirmation, Karakousis et al. (2003) argued the usefulness of polymorphic SSR markers for the discrimination of breeding material in Australian barley. In barley, important traits such as salt tolerance are controlled by polygenes with additive and dominant effects that are described by quantitative trait loci (QTL5) as salt tolerance is controlled by a variety of mechanisms (Eilles et al., 2000).

Varying marker response to salt stress indicates that some markers are more suitable for use in marker-assisted breeding than the other and that scssr0397 was the best in marker-assisted selection followed by Bmag770 and HVM67. These results are in a good harmony with those reported by (Eleuch et al., 2008; Chaabane et al., 2009; Aliyu et al., 2011). For the present 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. The productivity of SSR markers may be due to the possibility of amplification of the different size fragments from different regions of the genome or may be dependent on the genotypes, it clearly indicated that there were correlations among the salt tolerant genotypes. In general conclusion, It is clear from this study that the ability of plants to tolerate salt stress is determined by multiple physiological pathways on barley plants which grown under salinity stress at 6000 ppm, led to increases in the synthesis of osmotically active metabolites, amino acid proline and ABA such all these compounds might be used to protect the plants against stress conditions, on other way it was observed decreasing of Chlorophyll A and Chlorophyll B in the four cultivars under salt stress as compared to its content in plants grown under non salt stress condition. With respect to the molecular level analysis, the results showed high levels of polymorphism among the four Egyptian barley genotypes under salt stress included in this study, which refers to the high ability of SSR markers to reveal most of the information in a single locus and can be used for molecular genetic analysis at salinity stress tolerance on barley cultivars. However the observed results using SSR molecular markers may provide useful information on the history and biology of barley genotypes, but it does not necessarily reflect what may be observed with agronomic traits (Manifesto et al. 1999).

Table 1. The entry name, pedigree and degree of salt tolerance of the studies barley genotypes.

Genotypes	Degree Of Salt Tolerance	Origin	Pedigree
Giza 123	High Tolerant	Egypt	Giza 117/FAO 86 (Giza 117 = Baladi 16/Palestine 10)
Giza 124	Tolerant	Egypt	Giza 117/Bahteem 52// Giza 118/FAO 86
Giza 125	Tolerant	Egypt	Giza 117/Bahteem 52//Giza 118/FAO86
Giza 126	Tolerant	Egypt	WI 2291/4/11012-2/70-2245/3/Apam/IB65/A16

Table 2. Barley SSRs primers related to salt stress, their sequences, the chromosomal location (Von Korff et al., 2004) of derived loci, size range, marker type, motif and the reference

No	Marker	PCR primers	Chromosome	Size	Type	Reference
1	HVHOTR1	F:ATGAGCAGTCTTGTCTTAACC R:AGTTGGTCGCTAGATCTTATG	2H	165	SSR	Hayden et al. (2006)
2	HVM67	F:GTCGGGCTCCATTGCTCT R:CCGGTACCCAGTGACGAC	4H	116	SSR	Ramsy et al. (2000)
3	scssr0397	F: CTCCATCACACCATCTGTG R: GACATGGTTCCTTCTTCTC	5H	Unknown	SSR, SNP	Hearnden et al. (2007)
4	Bmac0316	F: ATGGTAGAGGTCCCAACTG R :ATCACTGCTGTGCTTAGC	6H	135	SSR	Ramsy et al. (2000)
5	Bmac0209	F: CTAGCAACTTCCCAACCGAC R:ATGCCTGTGTGGACCAT	3H	176	SSR	Varshney et al. (2007)
6	Bmag770	F: AAGCTCTTTCTGTATTTCGTG R: GTCCATACTCTTAACATCCG	1H	158	SSR	Ramsy et al. (2000)

Table (3). Abscisic acid concentrations (mg/100 g fresh weight) in leaves of the four barley (*Hordeum vulgare* L.) cultivars under control and 6000 ppm salt stress conditions.

Cultivars	Non Salt stress	Salt stress	Relative ABA content*(X-Folds)
1	48.60	59.60	1.22
2	33.12	125.17	3.78
3	25.16	167.65	6.66
4	13.12	154.45	11.77

* Relative ABA content = Treatment/ Control

Table (4). Effect of Salinity Stress Levels on Proline Concentration (200 mg/L) in leaves of the four barley (*Hordeum vulgare* L.) cultivars under control and 6000 ppm salt stress conditions.

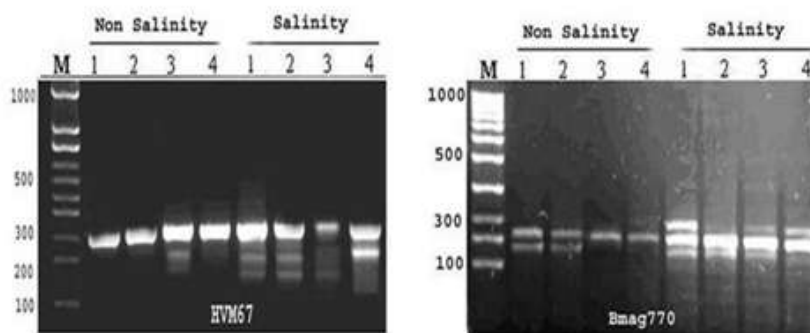
Cultivars	Non Salt stress	Salt stress
1	14.60	117.60
2	14.22	112.17
3	15.54	121.65
4	13.12	119.45

Table (5). Effect of Salinity Stress Levels on Chlorophyll a and b Concentration

Cultivars	Non Salt stress		Salt stress	
	Chlorophyll a	Chlorophyll b	Chlorophyll a	Chlorophyll b
1	1.37	0.74	0.82	0.53
2	1.44	0.81	0.86	0.59
3	1.54	0.85	0.88	0.65
4	1.85	0.92	0.95	0.85

Table (6). Barley SSR primers, their amplified fragments, polymorphic the polymorphism parentage and PIC value

Primer	Amplified fragments		Polymorphism %	pic
	Total (T)	Polymorphic		
HVHOTR1	4	1	25	0.754
HVM67	3	2	66	0.451
scssr0397	5	4	80	0.625
Bmac0316	3	3	100	0.525
Bmac0209	4	4	100	0.548
Bmag770	3	2	66	0.424



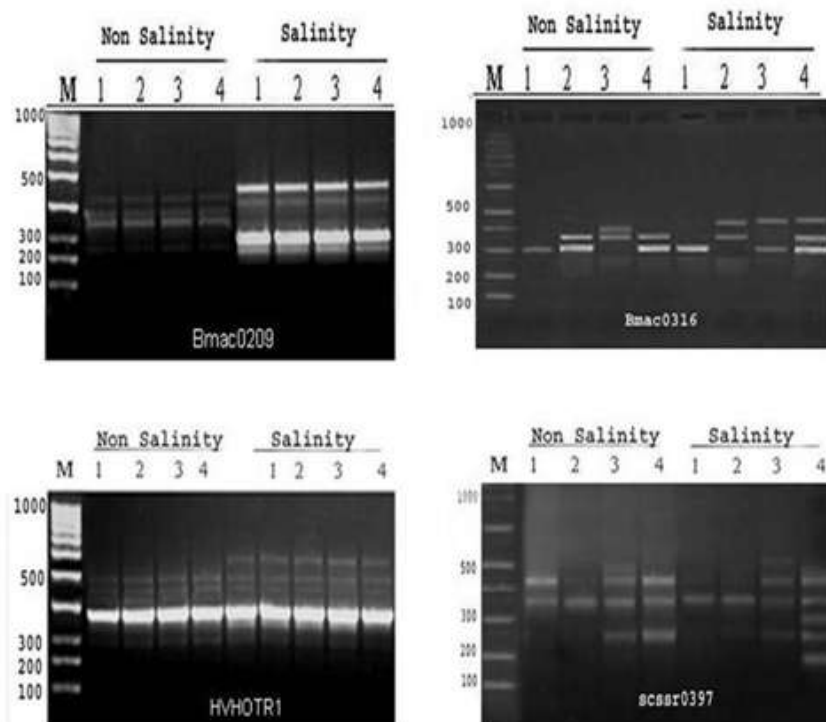


Fig. 1: PCR amplification profile generated from genomic DNA of four barley genotypes under salinity and non salinity stress with Six SSR primers, HVM67, Bmag770, Bmac0209, Bmac0316, HVHOTR1 and scsar0397. M-marker = 100bp 1-Giza 123, 2- Giza 124, 3- Giza 125, 4- Giza 126

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