Developing of QTL Associated with Salinity Tolerance in Chickpeas

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ABSTRACT

Soil salinity is one of the major abiotic stress which severely limits agricultural crop production throughout the world, and is increasing particularly in the irrigated agricultural areas as is the case in Egypt. Approximately, 33% of the irrigated land in Egypt is salt affected. Chickpea (Cicer arietinum L.) is an important grain legume that plays a significant role in the nutrition of the poor in the developing world. In this research, we used rich chickpea diversity panel collected from the GeneBank of the International Center for Agricultural Research in the Dry Area (ICARDA). This collection was selected by using the focused identification of germplasm strategy (FIGS). DNA of this core collection was extracted for molecular characterization using 16 SSR and 3 ISSR polymorphic markers. Association mapping was conducted by using Single-Locus-F test. The results indicated significant differences between the chickpea genotypes. Based on the average between the two locations, seven tolerant genotypes IGs (70275, 70249, 70351, 70782, 8447, 70330, 9434 and 70309) have been identified as tolerant genotypes. Association mapping indicated that SSR marker TAA170 was associated with salinity tolerance (P = 0.006201848). The results indicated that SSR marker TAA170 is close to the acidic endochitinase-like protein gene which is thought to be responsible for the salinity stress tolerance. This marker could be a candidate for marker assisted selection in chickpea.

Keywords: *Association mapping, Chickpea diversity, QTL, Salinity tolerance, Acidic endochitinase like protein gene.*

INTRODUCTION

It was reported that salinity affects about 100 million ha of arable lands worldwide where this area is expanding dramatically. Thus, developing tolerant crop varieties is critical (Vadez *et al.* , 2007 a and b). Upon exposure to salt stress, the meristems accumulate the salt in the vacuoles of the xylem (Parihar *et al*., 2015), to reduce their osmotic potential to reach –0.8 MPa (Flowers *et al.*, 2010). Salinity tolerance is observed in excluding ions from shoots and sequestering excess ions that arrive in leaves into vacuoles till reaching high concentrations (Jha *et al.* , 2014). Then, these ions either build up rapidly in the cytoplasm and inhibit enzyme activity causing cell death, or dehydrate the cell by accumulation in the cell walls (Parihar *et al*., 2015). To screen for new genes for tolerance to abiotic stresses, focused identification germplasm strategy (FIGS) is thought to be effective tool (Khazaei *et al*., 2013). To our knowledge, only two studies reported the

presence of QTLs for salinity tolerance (Pushpavalli *et al*., 2015). In a breeding population resulting from a cross between ICC4958 and Annigeri, TAA170 and STMS11 in linkage group 4 (LG4), were found to be associated with drought tolerance (Jha *et al*., 2014). Moreover, ICCM0249, NCPGR127, TAA170, NCPGR21, TR11, GA24 and STMS11 are seven SSR markers found to be related for drought tolerance. The two marker intervals (TAA170–NCPGR21) and (NCPGR127–TAA170) were found to be linked to drought tolerance-related traits (Varshney *et al*., 2014 a and b). No report on putative candidate genes, that would confer salinity tolerance in chickpea till date is conducted (Pushpavalli *et al*., 2015).

However, *Hot2* mutant of the Arabidopsis, which encode an endochitinase-like protein, was thought to be involved in salt stress tolerance. Moreover, when chitinases were over expressed in tobacco, it was resistant to salinity, pathogens and heavy metals. This is because when the plant is exposed to salt stress, the reinforcement of the outer most cell wall is enhanced. The metabolic processes which reinforce the outer most cell wall include carbohydrate degradation, glycoprotein processing and restructure of cellulose and hemicelllulose (Song *et al*., 2011). Another study (Kwon *et al*., 2007) revealed the role of acidic endochitinase in accumulation of high levels of NaCl in cells under either normal or NaCl stress conditions.

In the present study, the authors investigated essential lines and the diversity panel was established based on land races and wild accessions. The accessions used were thought to be salt stress tolerant because they were selected from saline environments by using the Focused Identification Germplasm Strategy (FIGS). Genotyping. The chickpea panel is also targeted for this study to investigate markers associated to salt stress trait. The aim of *in silico* PCR is to locate the tested markers on the chickpea genome.

MATERIALS AND METHODS

The studied germplasm panel is composed of 203 different genotypes that were collected from 28 provinces in 13 countries across the globe. The seeds were provided by the GeneBank of ICARDA using FIGS tool. The affectivity of FIGS is due to its combination of both agro-ecological information and data on plant characteristics. Most of the chickpea genotypes were from Pakistan and India provinces which are thought to have saline environments. The 138 genotypes were used in both the hydroponic and field environments.

Sowing the genotypes in the field and the green house

The experiment was performed in the field of Arish, Sainai, Egypt. Another experiment was performed in the green house by using the hydroponic system. In each year the individuals were replicated twice and arranged in a randomized complete block design. A drip water irrigation system was installed to water the pots every two weeks. In the green house, one seed of each individual accession was sown in tray (10cm x 30 cm) containing a mixture of peatmoss (40%) and perlite (60%). After two weeks of germination, the seedlings were transferred to hydroponic tank (150 cm x 230 cm). To determine the salt content in the soil of the field and the irrigation water, soil samples were air dried, softened and sieved prior to prepare soil paste, then soil solution was extracted to determine pH, soil salinity, cations and anions (Page, 1982). The salt concentration of the hydroponic tank was adjusted at 100 mM and the pH was adjusted at 8 (both were checked daily). Salinity stress tolerance trait was evaluated as the necrosis score. The phenotypic scaling was from 1 to 5, where the

tolerant plant was given score of 1, and the sensitive plant was given score of 5,and, when the plant was partialy tolerant, it was given number 2 ,3 or 4 according to the intensity of the necrosis. The phenotypic reading of the greenhouse was taken every three weeks during the experiment time. For the field experiments the phenotypic readings were taken every two months. Phenotypic scoring for genotypes of whole population was carried out in years 2014 and 2015. One way ANOVA was performed to test the significance of both the field and green house phenotypic data (Payne *et al.*, 2011).

DNA Extraction

0.2 g of fresh tissue were ground by liquid nitrogen using morter and pestle. 1 ml CTAB was added to the ground samples and mixed well to be incubated for 1 h at 65 ˚C. 1 ml chloroform: Isoamyl (24:1) was added to the samples and the mixture was shacken for 20 minutes. The samples were centrifuged for 15 min., and the supernatant was transferred to a new tube. 1 ml of absolute cold isopropanol was used to precipitate the DNA. The samples were then centrifuged. The resulting pellet was washed twice by 70% ethanol. The samples were airdried and eluted in 200 µl 1X TE (Rogers and Bendich, 1989).

Polymerase chain reaction (PCR)

PCR reactions were performed in 15 µl reaction volume consisting of 5 ng DNA template, 10 picomol of forward primer, 10 picomol of reverse primer, 0.1 U of MyTaq DNA polymerase, Bioline GmbH, Germany, 25 mM of $MgCl_2$, 2 Mm dNTPs and 10X PCR buffer in 96 -well micro titer plates using Applied biosystem thermal cycler, made in singapore. For the SSR markers, PCR program was used to amplify DNA fragments: initial denaturation was 5 min at 95°C. This was followed by initial 35 cycles of denaturation for 15 sec at 95°C, annealing for 15 sec at 55°C and extension for 30 sec at 72°C. Subsequently, 7 min final extension at 72°C. similar PCR profile was used for the ISSR markers, except for the annealing temperature, which was 45 ˚C. PCR products of SSR markers were checked for amplification on 9 % polyacrylamide gels due to its high resolution, and on 1.5% agarose gel for the ISSR markers.

The analysis was performed at Power marker (Liu and Muse, 2005). F-test was performed for each marker to test its linkage to the salinity stress tolerance. Polymorphic information content (PIC) value was calculated from the summary statistics tool by using the same software. Table (1) represents the references of the Polymorphic SSR markers used in this study. However, the ISSR markers used in this study were selected randomly. These markers were designed and synthesized by ICARDA lab at AGERI, Giza, Egypt. Three polymorphic ISSR markers were selected. The three markers are 805, HB12-N31 and ISSR-835.

***In silico* PCR**

FastPCR software (Kalendar *et al*., 2014) was used to find the location of the marker on the chickpea genome by in silico PCR. The map viewer tool in the NCBI database enables the user to know the location of the annotated genes on the genome. By combining the location of the target marker with the location of a candidate gene, the distance between the QTL and the gene was 1.2 megabase.

Phylogenetic analysis

By using power marker Frequency-based, distance was measured using shared allele method (Liu and Muse, 2005). The algorithm that was used to reconstruct the phylogeny from a distance matrix was Neighbor joining (Liu and Muse, 2005).

Table (1): List of the used SSR primers, which were selected from previous references.

Primer name	References
TA194	(Vadez *et al*., 2012) (Nayak, 2010) (Gujaria *et al*., 2011) (Samineni, 2010)
TA18	(Nayak, 2010) (Vadez *et al*., 2012)
TR31	(Gujaria *et al*., 2011) (Samineni, 2010) (Nayak, 2010)
TA37	(Vadez *et al*., 2012) (Samineni, 2010) (Nayak, 2010)
ICCM0178	No review
ICCM0293	No review
TAA170	(Jaganathan, 2015) (Samineni, 2010) (Rahman, 2009)
TR29	(Nayak, 2010)
H1B09	(Hamwieh *et al*., 2013) (Hiremath *et al*., 2012)
TA179	(Hamwieh *et al*., 2013) (Pushpavalli *et al*., 2015) (Gujaria *et al*., 2011) (Hiremath *et al*., 2012)
TA42	(Nayak, 2010) (Pushpavalli *et al*., 2015) (Samineni, 2010)
TA71	(Vadez *et al*., 2012) (Nayak, 2010) (Gujaria *et al*., 2011) (Hiremath *et al*., 2012)
TAASH	(Nayak, 2010)
ICCM0293	No review
TR7	(Nayak, 2010) (Rahman, 2009) (Samineni, 2010)

RESULTS AND DISCUSSION

Chickpea is widely grown in West and Central Asia and Australia, where saline soils are abundant. There is a narrow genetic variation among different genotypes, which is an obstacle for breeding for salinity tolerance. Moreover, there is difficulty in how to evaluate salinity tolerance and whether to approach salt stress research from the field or from the lab (Vadez *et al.,* 2007 a). In chickpea, despite the conductance of several mapping studies, only two studies have reported the presence of QTLs for salinity tolerance. Similar results were reported earlier by Jaganathan *et al.* (2013), Jha *et al.*, (2014) and Varshney *et al.* (2014 a and b) where a putative QTL region was found for drought stress tolerance trait on CaL G04 in chickpea. The literature has affirmed that plant responses to salt and water stress have much in common (De Oliveira *et al.* , 2013) . There is no report

on putative candidate genes that would confer salinity tolerance in chickpea (Pushpavalli *et al.* , 2015).

Soil analysis of field experiment in Arish

The salt concentration in the field was 344 ppm in the first 30 cm depth, 904 ppm from 30 to 60 cm depth, and 848 ppm at more than 60 cm depth. The analysis of the irrigation water revealed that the average salt concentration was 897 mM, and the pH was 7.2.

The phenotypic data

As deduced from Fig. (1), the plant which scores reading till 3.25 was considered to be tolerant, the plant which scores (5) as necrosis scale was considered sensitive to death. 47 accessions were observed to be tolerant. 3 accessions were sensitive to death.

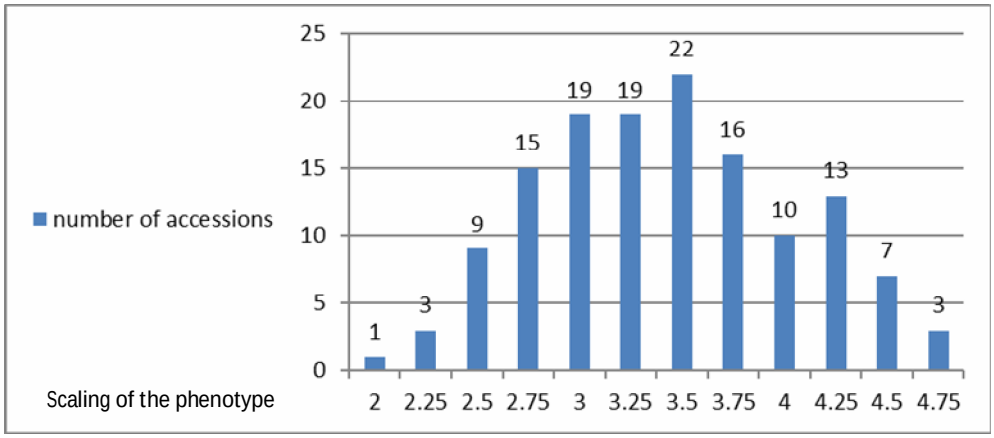

Fig. (1): Histogram represents the number of associations for each scale of the phenotype.

Table (2): The origin and province of the salt tolerant genotypes.

Best genotype	Field data	Hydroponic data	Origin	Province
IG70782	2	2	Pakistan	Punjab
IG70430	2.5	2	Pakistan	Punjab
IG70764	2.5	2	Pakistan	Sindh
IG117703	2.5	2	Pakistan	Punjab
IG6057	2.5	2.5	Pakistan	NWF
IG8447	2.5	2.5	Azerbaijan	Lankaran
IG70249	2.5	2.5	Pakistan	Sindh

Table (2) represents the most tolerant genotypes obtained by analysis of variance (ANOVA) from the phenotypic readings of the green house and the field. It was deduced from the table that among the seven most tolerant genotypes, six were from Pakistan, and one from Azerbaijan. CV% = 7.8; LSD = 1.27; *** indicate significance for the result, d.f. stands for degree of freedom, SS stands for sum of squares, MS stands for mean square and F stands for the F- test. 138 genotypes were screened in the field and the green house

(Arish Egypt and hydroponic). 100 mM NaCl was used in hydroponic method. No significant difference were observed between the two locations (Table 3). Highly significant differences were identified between the genotypes.

It was deduced from Table (2) that the tolerant genotypes were from Pakistan and Azerbaijan. Phenotypic evaluation exhibited significant variations for salinity stress tolerance under saline conditions between various genotypes indicating a broad genetic and phenotypic variance within the examined chickpea population. The present data indicated the presence of genetic components influencing salinity tolerance trait. Phenotypic evaluations were made across the years 2014 and 2015 through field and greenhouse experiments.

Table (3): The Analysis of variance table for the hydroponic and the field experiments of the salinity tolerance trait.

Source of variation	d.f.	SS	MS	F
Genotype in 2014 and 2015	137.00	210.88	1.54	1.86***
Location	1.00	1.04	1.04	1.26
Genotype X Location	137.00	150.46	1.10	1.33
Error	275.00	227.41	0.83	
Total	550.00	589.77		

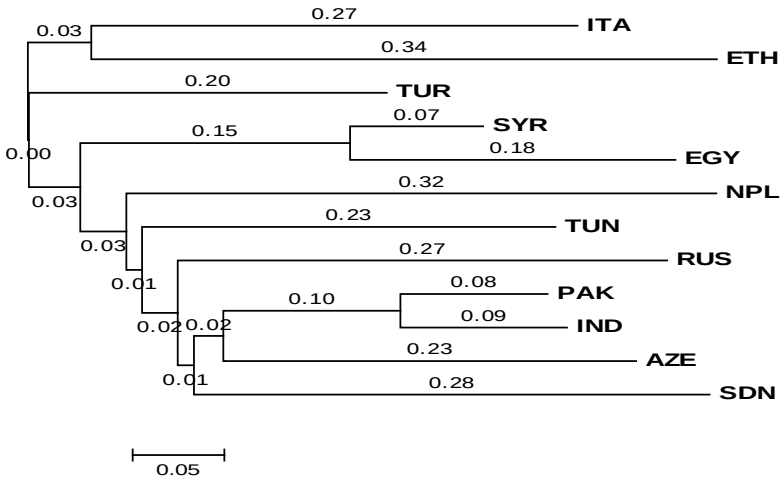

Fig. (2): Phylogenetic tree representing the relation between the genotypes. The origin of the genotypes is represented as the branch name. The branch lengths is represented by numbers for each taxon. ITA stands for Italy, ETH stands for Ethiopia, TUR stands for Turkey, SYR stands for Syria, EGY stands for Egypt, NPL stands for Nipal, TUN stands for Tunisia, RUS stands for Russia, PAK stands for Pakistan, IND stands for India, AZE stands for Azrabijan, SDN stands for The Sudan.

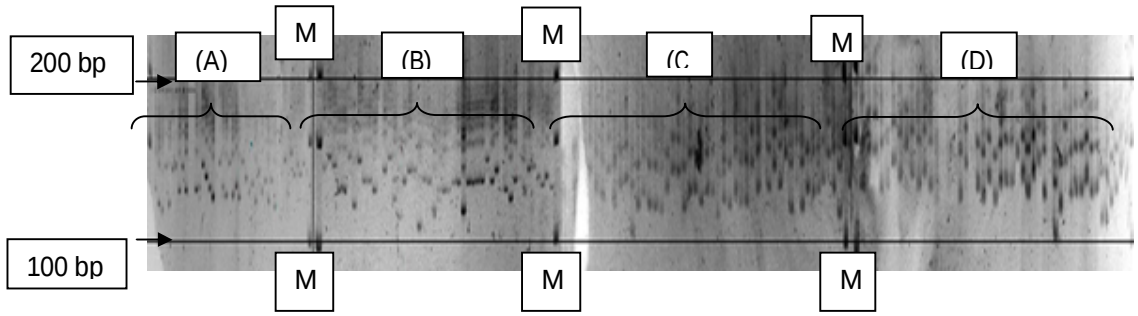

Fig. (3): 9 % polyacrylamide gel illustrating TAA170 SSR PCR product separation . 192 samples are represented in the figure. (A) represents samples from 1 to 48. (B) represents samples from 49 to 96. (C) represents samples from 97 to 144. (D) represents samples from 145 to 192. (M) represents the 100 bp ladder (Vivantis). The alleles of the polymorphic TAA170 are of different product sizes. Scoring of the bands in this gel was done manually, from which the p-value and the PIC value was calculated. TAA170 is a polymorphic SSR marker whose PIC value was 0.9. This marker was found to be associated to the salinity tolerance trait after calculating its p-value which was found to be 0.006 from the hydroponic experiment, and 0.06 from the field experiment.

The origin of the genotypes is represented as the branch name. ITA stands for Italy, ETH stands for Ethiopia, TUR stands for Turkey, SUR stands for Syria, EGY stands for Egypt, NPL stands for NPL, TUN stands for Tunisia, RUS stands for Russia, PAK stands for Pakistan, IND stands for India, AZE stands for Azrabejian and SDN stands for The Sudan. The branch lengths is represented by numbers for each taxon.

GWAS-QTL detection and quantification

The present Genomic Wide Association Mapping (GWAS) detected one QTL for salinity tolerance trait. Only one QTL was detected because we used only 16 polymorphic SSR markers that were known in the literature to be linked to salinity tolerance as shown in Table (1), besides using 3 polymorphic ISSR.

The significance of the linkage of the markers to the salinity stress tolerance was detected from their p-value to get rid of the false positive QTL effect. The P-value of TAA170 was 0.006201848, which indicates the role of a major gene controlling tolerance. The linked marker was found on chromosome 4, in region from 13040128 to 13040501. The PIC value detect the ability of the marker to find the genetic variation among the used chickpea diversity set. The phylogenetic tree indicated the clustering of genotypes of close origins. Similar results were reported earlier by Jaganathan *et al.* (2013), Jha *et al.* (2014) and Varshney *et al.* (2014 a and b) where a putative QTL region was found for drought stress tolerance trait on CaL G04 in chickpea. The literature has affirmed that plant responses to salt and water stress have much in common (De Oliveira *et al.*, 2013).

Table (4): The p-value and the PIC values of the used SSR primers in the hydroponic and field experiments.

PIC	Marker	Hydroponic data	Arish field data	Average data of both experiments by ANOVA
0.5	ICCM0178	1.0	0.7	0.4
0.9	TAA170	0.006	0.06	0.02
0.3	H1H13	0.6	0.8	0.8
0.5	H1B06	0.3	0.4	0.9
0.7	TA194	0.3	0.4	0.07
0.5	TA37	0.2	0.6	0.3
0.8	TA18	0.8	0.1	0.6
0.7	ICCM0293	0.9	0.4	0.9
0.8	TA42	0.2	0.4	0.6
0.6	TR31	0.5	0.9	0.4
0.7	TA179	1.0	0.4	0.9
0.8	TR29	0.52	0.2	0.2
0.7	TA71	0.2	0.4	0.1
0.6	TAASH	0.4	0.7	0.5
0.7	H1B09	0.1	0.4	0.6
0.6	TR7	0.6	0.6	0.5

Table (4) represents the 16 polymorphic SSR markers used and their association to the salinity stress tolerance in the hydroponic experiment in the green house and Arish field experiments. The average of the phenotypic readings of the hydroponic and the field experiments, calculated by ANOVA, were used. TAA170 showed constant and significant p-values in the both environments .

Table (5): The p-value and the PIC values of the used ISSR primers in the hydroponic and field experiments.

ISSR markers	Hydroponic	Field	Average data of both experiments by ANOVA	PIC value
807-250bp	1.0	0.9	0.9	0.08
807-650 bp	0.9	0.4	0.4	0.1
807-750bp	0.6	0.3	0.3	0.3
HB12-N31-1000bp	0.9	0.8	0.8	0.4
HB12-N31-1050bp	0.7	0.5	0.5	0.3
HB12-N31-1100 bp	0.4	0.5	0.5	0.4
HB12-N31-1150 bp	0.6	0.5	0.5	0.3
HB12-N31-1200	0.8	0.4	0.4	0.4
HB12-N31-1250bp	1.0	1.0	1.0	0.2
HB12-N31-1300bp	0.2	0.9	0.9	0.2
HB12-N31-1350bp	0.1	0.3	0.3	0.4
HB12-N31-250bp	0.8	0.6	0.6	0.1
HB12-N31-300bp	0.8	0.8	0.8	0.4
HB12-N31-350 bp	0.7	0.3	0.3	0.4
HB12-N31-400bp	0.1	0.09	0.09	0.4
HB12-N31-500 bp	0.8	0.8	0.8	0.4
HB12-N31-600bp	0.3	0.3	0.3	0.3
HB12-N31-700bp	1.0	0.4	0.4	0.3
HB12-N31-800bp	1.0	0.8	0.8	0.4
HB12-N31-900 bp	0.9	1.0	1.0	0.4
ISSR-835-350bp	0.7	0.6	0.6	0.5
ISSR-835-500 bp	0.6	0.3	0.3	0.5
ISSR-835-620 bp	0.4	0.6	0.6	0.5
ISSR-835-700 bp	0.6	0.2	0.2	0.3
ISSR-835-900bp	0.9	0.5	0.5	0.4

Table (5) represents the p-values of the ISSR markers and their PIC values in both the hydroponic experiment in the green house and the field. The average of the phenotypic readings of the hydroponic and the field experiments, calculated by ANOVA, were used.

It was concluded that there was no association with any of the ISSR with the salinity stress tolerance. As observed from the phylogenetic tree (Fig.2), the genotypes from close locations are similar.

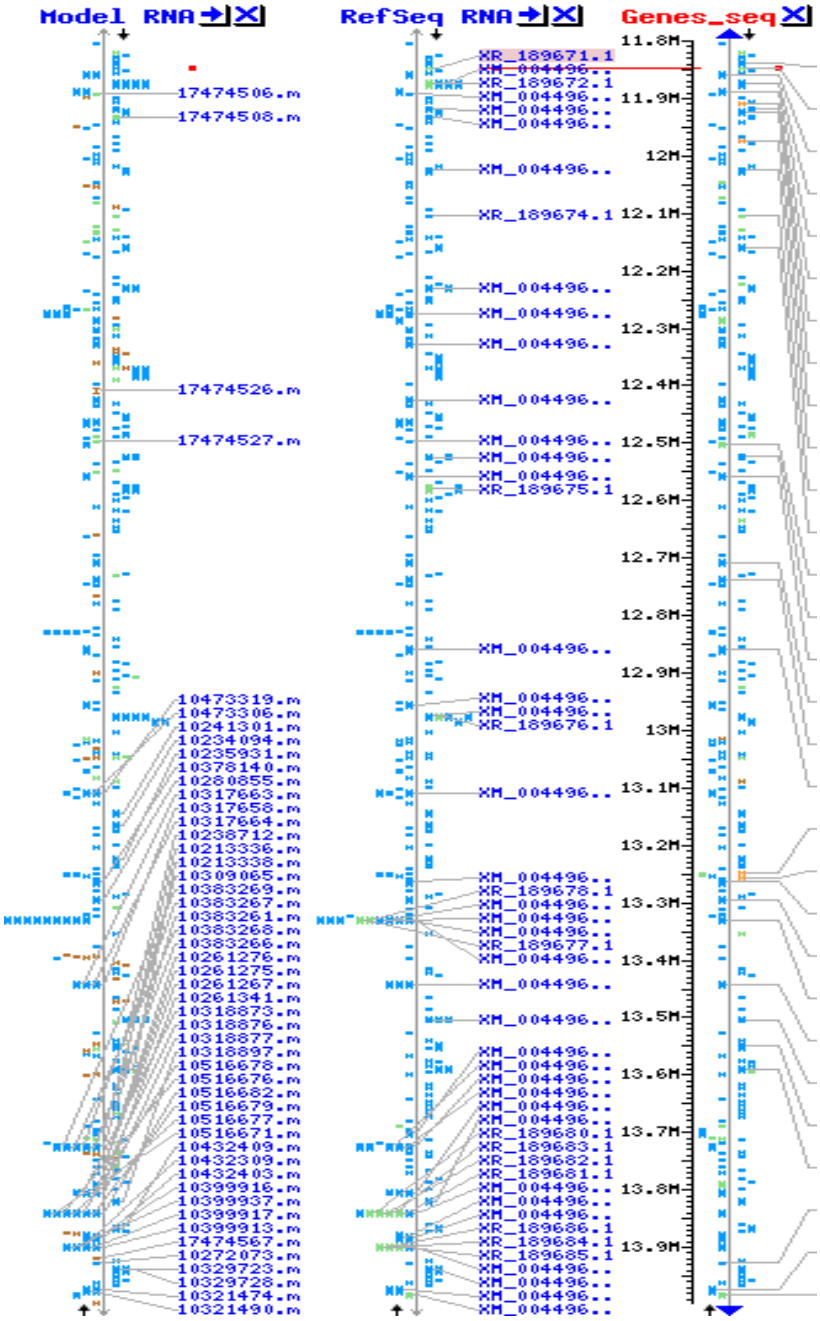

Fig. (4): The location of the marker (TAA170) relative to the location of acidic endochitinase-like protein gene. The QTL was detected on chromosome 4 at 13040128 base to 130040501 base. The position of the neighboring gene, Acidic endochitinase, is on chromosome 4 from 11847137 base to 11848288 base.

In this research 70275, 70249, 70351, 70782, 8447, 70330, 9434 and 70309 have been identified as salt tolerant genotypes. The association mapping indicated that SSR marker TAA170 was associated with salinity tolerance (P = 0.006201848). The results indicated that SSR marker TAA170 is close to the Acidic endochitinase- like protein gene which is thought to be responsible for the salinity stress tolerance. This marker could be a candidate for marker assisted selection in chickpea.Thus, this study revealed the prescence of a putative QTL located on chromosome 4. The QTL was detected on chromosome 4 at 13040128 base to 130040501 base. The position of the neighboring gene, Acidic endochitinase, is on chromosome 4 from 11847137 base to 11848288 base as shown in Fig. (4). In this research, using the map viewer tool of NCBI, an essential acidic endochitinase gene was found. Acidic endochitinase gene is known for its role in the reinforcement of the cell wall, thereby enhancing the physical barrier against deleterious salt stress (Song *et al.*, 2011). It is known that two proteins are expressed from this gene which are GH18_hevamine_XipI_class_III and Hevamine. GH18_hevamine_XipI_class_III is a conserved domain family that includes xylanase inhibitor Xip-I, and the class III plant chitinases such as hevamine, concanavalin B, and PPL2, all of which have a glycosyl hydrolase family 18 (GH18) domain. Hevamine is a class III endochitinase that hydrolyzes the linear polysaccharide chains of chitin and peptidoglycan and is important for defense against pathogenic bacteria and fungi. PPL2 (Parkia platycephala lectin 2) is a class III chitinase from Parkia platycephala seeds that hydrolyzes beta (1-4) glycoside bonds linking 2-acetoamido-2-deoxy-beta-D-glucopyranose units in chitin (Geer *et al.*, 2009). To enhance the physical barrier against salt stress there are changes in the cell wall related proteins. Many cell wall metabolic processes such as carbohydrate degradation, glycoprotein processing and restructure of celluloseand hemicelllulose ultimately lead to the reinforcement of the cell wall. Post-translational modifications of the protein is a possible reason resulting in different responses to salt stress (Song *et al.*, 2011). Moreover, it was observed by Kwon *et al.*, (2007) that mutants of hot2 had an accumulated high levels of NaCl in cells under either normal or NaCl stress conditions.

CONCLUSION

The salt tolerance associated SSR marker, TAA170 can be used to select salinity tolerant genotypes in marker-assissted selection. The neighboring gene locus which is Acidic endo chitinase protein-like gene, is known to be responsible for salinity tolerance.

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الملخص العربي

تحديد واسمات جزيئيه مصاحبه لتحمل الملوحه في الحمص

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تعتبر ملوحه التربه واحده من اكبر المؤثرات الغيرحيويه التي تؤثر بشده علي الانتاج الزراعي في العالم و هذا المؤثر يتزايد في الاراضي المرويه. قرابه ٣٣% من الاراضي المرويه متأثره بملوحه التربه . الحمص من اهم الحبوب البقوليه التي تلعب دورا هاما في تغذيه الفقراء في الدول الناميه. استخدمت اصناف متنوعه من الحمص من بنك الجينات في ايكاردا، سورية. تم تجميع الاصناف بطريقه متطوره تسمي ال"FIGS". تم اختبار ١٣٨ صنف في الحقل في العريش و الصوبه البلاستيكيه. صممت التجربه بمكررين لكل صنف و بطريقه مصفوفة الفا. تركيز الملوحه (كلوريد الصوديوم) في تجربه الصوبه كان ١٠٠ ميللي مولر بينما كان ٣.٠٠ ميللي مولر في اول ٣٠ سم عمق و ١.١٣ ميللي مولر في عمق من ٣٠ سم -٦٠ سم و ١.٠٦ ميللي مولر في عمق اكبر من ٦٠ سم. تم استخلاص الحمض النووي من اصناف الحمص المتنوعه و تم تصنيفهم جزيئيا بواسطه ١٦ واسم وراثى" SSR " و ثلاث واسمات " ISSR". انتقاء واسمات ال SSR كان علي اساس ارتباطهم بتحمل الملوحه في أبحاث اخري. تم رسم الخريطه الوراثيه عن طريق " Single locus F-test". اقرت النتائج وجود فروقات واضحه بين اصناف الحمص. تم التحليل الاحصائي بواسطه قراءات الصوبه و الحقل حيث كان السبع اصناف الاكثر مقاومه (70275, 70249, 9434 70330, 8447, 70782, 70351و70309). بعد رسم الخريطه الوراثيه تم التعرف علي واسم " TAA170" كمؤشر مرتبط بتحمل الملوحه. اكدت النتائج علي وجود جين يسمي " acidic-chitinase protein like protein" بالقرب من الواسم "TAA170" . هذا الجين من المحتمل ان يكون مسئول عن تحمل الملوحه. اما بالنسبه للواسم "TAA170" فيمكن استخدامه لتحسين اصناف الحمص فيما بعد.