

CLONING SOME SALINITY STRESS RELATED GENE(S) IN BARLEY

By

MONA IBRAHIM MOHAMMED IBRAHIM

B.Sc. Agric. Sci. (Genetics), Ain Shams University (1999)

M.Sc. Agric. Sci. (Genetics), Ain Shams University (2004)

A thesis submitted in partial fulfillment

of

the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Agricultural Science

(Genetics)

Department of Genetics

Faculty of Agriculture

Ain Shams University

2010

Approval Sheet

**CLONING SOME SALINITY STRESS RELATED
GENE(S) IN BARLEY**

By

MONA IBRAHIM MOHAMMED IBRAHIM

B.Sc. Agric. Sci. (Genetics), Ain Shams University (1999)

M.Sc. Agric. Sci. (Genetics), Ain Shams University (2004)

This thesis for Ph.D. degree has been approved by:

Dr. Ashgan El-Sayed Abou Gabal

Prof. of Genetics, Department of Plant, Faculty of Agriculture,
Saba Basha, Alexandria University

Dr. Khaled Abdelaziz Soliman

Prof. of Genetics, Department of Genetics, Faculty of Agriculture,
Ain Shams University

Dr. Aly Z.A. Abdelsalam.....

Prof. of Genetics, Department of Genetics, Faculty of agriculture,
Ain Shams University

Dr. Samir Abdelaziz Ibrahim.....

Prof. Emeritus. of Genetics, Department of Genetics, Faculty of
Agriculture, Ain Shams University

Date of Examination: 22 /11 / 2010

CLONING SOME SALINITY STRESS RELATED GENE(S) IN BARLEY

By

MONA IBRAHIM MOHAMMED IBRAHIM

B.Sc. Agric. Sci. (Genetics), Ain Shams University (1999)

M.Sc. Agric. Sci. (Genetics), Ain Shams University (2004)

Under the supervision of:

Dr. Samir Abdelaziz Ibrahim

Prof. of Genetics, Department of Genetics, Faculty of
Agriculture, Ain Shams University (Principal Supervisor)

Dr. Aly Z.A. Abdelsalam

Prof. of Genetics, Department of Genetics, Faculty of agriculture,
Ain Shams University

ACKNOWLEDGEMENT

First, of all my obedience, devotion, deepest thanks and praise are due and fully extended-as always to **Allah, the greatest and almighty** who has created us and bestowed upon us a lot of blessing which we cannot enumerate and thank enough.

I would like to express my deepest thanks and sincere gratitude to Prof. **Dr. Aly Z.A. Abdelsalam**, Professor of Genetics, Genetics Dept., Ain Shams University for his supervision, suggesting the problem valuable advices, unfailing help during the course of investigation and in writing the manuscript. This work benefited greatly from his efforts.

Great thanks would be expressed to **Prof. Dr. Samir A. Ibrahim** Professor of Genetics, Genetics Dept., Ain Shams University, for his kind supervision. Sincerely, no word can explain my deep feeling of gratitude for his valuable suggestion and great support along this study.

I would like to extend my deepest gratitude to Prof. **Dr. Ahmed Bahieldin Mohamed**, Professor of Genetics, Genetics Dept., Ain Shams University, for his kind supervision, suggesting the problem, unlimited support.

Special thanks are due to **Dr. Hala Eissa**, Senior researcher of the ESL lab, Agricultural Genetic Engineering Research Institute (AGERI) for providing the laboratory facilities for this study and writing the manuscript.

Special thanks are due to **Dr. Sabah M. Hassan**, Associate professor, Genetics Department, Ain Shams University for writing and reviewing the manuscript. In addition, special thanks to all the staff members of the ESL lab, Agricultural Genetic Engineering Research Institute (AGERI), for their great help during my work specially, **Dr. Ahmed Shokry**

I am deeply indebted to my friends and my family; my father my mother and my sisters **Naglaa** for their love, support and their continuous encouragement and praying for me throughout my life.

ABSTRACT

Mona Ibrahim Mohammed Ibrahim, Cloning Some Salinity Stress Related Gene(S) In Barley. Unpublished Ph. D. Thesis. Genetic Department, Faculty of Agriculture, Ain Shams University, 2010

The main task of the present work was to isolate and characterize abiotic stress-related gene(s) from barley (*Hordeum spontaneum* L.) through polymerase chain reaction (PCR). Two salt-responsive cDNA fragments were characterized *via* differential display-polymerase chain reaction (DDR-PCR) from wild barley (*Hordeum spontaneum* L.) under salt stress (250mM NaCl) were pre selected as the most tolerant against salt stress (**Eissa *et al.*, (2007)**). Polymerase chain reaction (PCR) was adopted to amplify *RAP1/4* and *SCP11/1* related sequences using specific primers and genomic DNA isolated from wild barley samples. PCR amplified products were purified, cloned, transformed into *E. coli* and sequenced using ABI PRISM 310 Gene Analyzer. DNA sequencing were detected and then subjected to homology searching *via* computer software. The results indicated that the fragment of RAP ¼ B contained many conserved domains and its nucleic acid sequence was similar to the *DREB1/CBF3* from barley (*Hordeum vulgare* L.) with significant homology of 84%. Semi-quantitative RT-PCR showed that *HsCBF3* was induced in the Barley by NaCl (250 mM) treatments. These results suggested that the novel *CBF3* gene might play an important role in response to high salinity through binding to the DRE *cis*-element.

Key words: Barley, *Hordeum spontaneum* L., salt stress, cloning, function analysis, Semi quantitative-PCR, transcription factor, CBF3 gene.

LIST OF ABBREVIATION

ABA	Absciscic acid
ACP	Annealing-control-primer
Blast	Basic local alignment search tool
CBF	C-repeat binding factors
Cd	Conserved domain
DREB	Dehydration response element binding protein
FAO	Food and Agriculture Organization
HS	<i>Hordeum spontaneum</i>
HV	<i>Hordeum vulgare</i>
LEA	Late embryogenesis abundant
MCS	Multiple cloning site
RT-PCR	Reverse transcriptase-polymerase chain reaction
ROS	Reactive Oxygen Specie

CONTENTS

	Page
LIST OF TABLES	IV
LIST OF FIGURES	V
LIST OF ABBREVIATION	VII
I. INTRODUCTION	1
II. REVIEW OF LITERATURE	4
1. Barley importance	4
2. Plant abiotic stress	5
3. Salt stress	7
3.1. Salinity effects on plants	10
3.2. Plant response to salt stress	11
3.3. Genes expressed under salt stress	14
4.1. Regulatory proteins	17
4.1.1. Absciscic acid	17
4.2. Transcription factor	19
4.2.1. DREB transcription factor	19
III. MATERIALS AND METHODS	27
1. Material	27
1.1. Plant materials	27
1.2. Sequence collection	27
2. Method	27
2.1. Molecular genetic studies	27
2.1.1. Primer design	28
2.1.2. Primer preparation and dilution	28
2.2. DNA Extraction	28
2.3. Polymerase chain reaction (PCR) analysis	30
2.4. DNA electrophoresis	30
2.4.1. Agarose gel electrophoresis	30
2.4.2. Photography of DNA in agarose gel	31
2.5. Purification of PCR-amplified DNA	32

2.6. Cloning of PCR-amplified DNA gene.	33
2.6.1. Cloning vector	33
2.6.2. DNA ligation	34
2.6.3. Preparation of competent cells	34
2.6.4.. Transformation of competent cells	35
2.6.5. Miniprep of plasmid DNA	36
2.6.6.1. Screening of recombinants by PCR	37
2.7. Sequencing of PCR-amplified DNA	37
2.8. Identification of candidate genes using NCBI database	38
2.9. Plant material treatment	38
2.10. RNA extraction	38
2.10.1. DNase treatment	39
2.10.2. Spectrophotometric determination of RNA concentration	39
2.10.3. RNA electrophoresis	40
2.10.4. Reverse transcription of mRNA	41
2.10.5. Semi-quantitative RT-PCR analysis	42
IV. RESULTS AND DISCUSSION	43
1. Isolation RAP1/4 and SCP11/1 related genes from <i>Hordeum Spontaneum</i> genome	43
1.1. Amplification of RAP1/4 and SCP11/1	43
2.2. Cloning of RAP1/4 and SCP11/1	44
2.2.1. Screening of transformed bacterial cells	46
2.2.1.1. Blue/white colony screening	46
2.2.1.2. PCR screening of the recombinant clones	46
3. Sequencing of RAP1/4 and SCP11/1 related gene	49
4. In-silico analysis	54
4.1. Blast analysis	54
4.1.1. DNA sequence alignment of <i>RAP1/4A</i> fragment	54
4.1.2. DNA sequence alignment of RAP1/4B fragmen	54
4.1.3. DNA sequence alignment of SCP11/1 fragment	54
5. Characterization of RAP1/4B-related gene	57

III

5.1. Alignment of nucleic acid of RAP1/4B and CBF3	57
5.2. Identification of <i>CBF3</i> chromosomal location	59
5.3. Restriction map of <i>CBF3</i>	59
5.4. Functional analysis of cDNA (RAP1/4B) encoding transcription factor of CBF 3 gene	61
5.4.1. Salinity treatment	61
5.4.2. RNA electrophoresis	62
5.4.3. Check for the presence of the right specific genes products (CBF3 and Actin)	63
5.4.4. Expression patterns of CBF3 in leaves	63
V. SUMMARY AND CONCLUSION	65
VI. REFERENCES	68

LIST OF TABLES

No.		Page s
(1)	Names of barley genotypes and their origins	27
(2)	Transcript profile of salt-stressed barley (<i>Hordeum spontaneum</i> L.). S= Shoot, R= Roo	27
(3)	Sequence of the specific primers for RAP1/4 and SCP11/1 genes.....	28
(4)	Cloned fragments and homology results using NCBI database.....	53
(5)	The restriction enzymes, which can cut within the HVCBF3 gene and its exact position within the gene	61

LIST OF FIGURES

No.		Pages
(1)	pGEM®-T Easy Vector circle maps.....	34
(2)	Agarose gel electrophoresis of PCR products amplified from barley genomic DNA using RAP ¼ specific primers	45
(3)	Agarose gel electrophoresis of PCR products amplified from barley genomic DNA using SCP11/1 specific primers.....	46
(4)	Agarose gel electrophoresis (1%) showing candidate band after purification of PCR products amplifies. (M) 1kb marker. (A); showed RAP1/4A.. (B); showed RAP1/4B.....	47
(5)	Agarose gel electrophoresis showing candidate band after purification of PCR products. (M) 1kb (A); showed SCP11/1A, (B); showed SCP11/1B.....	48
(6)	Screening of inserted fragment of (RAP1/4) using PCR with M13 primer, where (M): 1.0 kb ladder DNA marker, (lanes 1:5) RAP1/4A.; (lanes 6 :10): RAP1/4B recombinant plasmid	48
(7)	Screening of inserted fragment of (SCP11/1) using PCR with M13 primer, where (M): 1.0 kb ladder DNA marker, (lanes 1:5): SCP11/1A, (lanes 6 :10): SCP11/1B recombinant plasmid	49
(8)	Nucleotides sequences of RAP1/4A	50
(9)	Nucleotides sequences of RAP1/4B.....	51
(10)	Nucleotides sequences of SCP11/1.....	52
(11)	Alignment and positions of RAP ¼ A fragment after sequencing using SGD database.....	55
(12)	Alignment and positions of RAP ¼ B fragment after sequencing using SGD database.....	56

(13)	Alignment and positions of SCP11/1 fragment after sequencing using SGD database.....	56
(14)	Alignment of nucleic acid of RAP1/4B and CBF3. The colored background indicate the well conserved nucleotides.....	58
(15)	Location map of CBF3 gene	60
(16)	Formaldehyde agarose gel electrophoresis of total RNA isolated from barley genotypes after different exposure periods (0, 1, 10, 72h and week) of salinity treatment.....	63
(17)	Comparison of <i>cbf3</i> expression patterns of barley shoots obtained by semi-quantitative RT-PCR of wild barley (H. Spontaneum) after salt treatments for 1h, 10h, 72h and week and the control (0 h). (B) Barley <i>Actin</i> gene was used as control for relative amount of RNA.....	64

I. INTRODUCTION

Barley (*Hordeum vulgare* L.) is one of the main cereals of the Mediterranean agriculture belt and is a founder crop of old world Neolithic food production and one of the earliest crops domesticated (**Harlan and Zohary, 1966; Mohammed and Takao 2007**).

Barley (*Hordeum vulgare* L.) is the fourth important cereal crop in Egypt after wheat, maize and rice (**Salem et al., 2009**). Barley is the main crop grown on a large scale in the low rainfed northern coastal region (100-200 mm annual rainfall), in newly reclaimed land and in regions affected by salinity or where irrigation water is limited. It is grown in both rainfed and irrigated conditions, though in the more favourable irrigated soils of the Nile Valley, barley gives way to more valuable crops. Barley production is severely affected by rainfall, as a consequence, the total barley area fluctuates dramatically in the world, e.g. 34,000 hectares (ha) in 1970-1974 to 188,000 ha in 1994/1995 (**Forster et al., 2004**).

Drought, high-salt and low-temperature stresses are important factors affecting the development of plants and the yields of crops. When exposed to these abiotic stresses, plants will undergo a series of physiological and biochemical changes, which lead to the activation or inactivation of some specific endocellular signaling pathways in response to stimuli (**Shinozaki and Yamaguchi-Shinozaki, 1996, Liu et al., 1998**). There are mainly two sorts of genes involved in these processes. Some encode proteins that can either participate in protecting plant cells from the stresses or repair injuries caused by the stresses, and the others are the protein kinases that respond to and transduce the stress induced signals or transcription activators (**Shinozaki and Yamaguchi-Shinozaki, 1996**). Salinity affects approximately 20% of the world's arable land and approximately 40% of irrigated land to various

degrees (**Sahi et al., 2006**). High salinity is one of the most important environmental stresses that can lead to changes in plant growth and development. Severe stress may threaten survival and finally undermine crop productivity. Improving the response of crop plants requires a thorough understanding of the cellular mechanisms that allow certain species to grow at high salinity. Plants respond to abiotic stress by altering the expression of many genes, which in turn affect major mechanism leading to adaptation and survival during periods of stress (**Hasegawa et al., 2000**). Salt-stress response is shown to encompass large number of genes including those linked to different pathways, which leads finally to a response on the whole plant level.

CBF/DREB (C-repeat binding factor/dehydration responsive element binding factor) family of transcription factors in plants is reported to be associated with regulation of gene expression under stress conditions.

Many genes that respond to drought, high-salt and low-temperature conditions were discovered to have DRE or CRT (C-repeat element) in their promoter regions (**Yamaguchi-Shinozaki and Shinozaki, 1994; Thomashow, 1999; Shinozaki and Yamaguchi-Shinozaki, 2000**). Five transcription activators, *i.e.* DREB1 A-C and DREB2 A-B, were obtained by screening the Arabidopsis cDNA library and by using the yeast one-hybrid method, which specifically bind to DRE and regulate the expression of stress-resistant genes. DREB1s are induced by low-temperature, whereas DREB2 is induced by drought and high-salt conditions. DREB1 and DREB2 independently function as transcription activators in low-temperature and drought-induced signaling pathways, respectively (**Liu et al., 1998**)

Furthermore the dehydration-responsive transcription factors (DREB) and C-repeat binding factors (CBF) bind to DRE and CRT

cis-acting elements that contain the same motif (CCGAC). Members of the CBF/DREB1 family, such as CBF1, CBF2, and CBF3 (or DREB1B, DREB1C, and DREB1A, respectively) are themselves stress-inducible. DREB/CBF proteins are encoded by AP2/EREBP multigene families and mediate the transcription of several genes such as rd29A, rd17, cor6.6, cor15a, erd10, kin1, kin2 and others in response to cold and water stress (**Ingram and Bartels 1996; Stockinger *et al.*, 1997; Gilmour *et al.*, 1998; Liu *et al.*, 1998; Seki *et al.*, 2001; Thomashow *et al.*, 2001**).

Barley (*Hordeum vulgare*) Cbf3 is located on barley chromosome 5H between markers WG364b and saf1p58 on the barley cv Dicktoo _ barley cv Morex genetic linkage map. This position is some 40 to 50 cM proximal to the winter hardiness quantitative trait locus that includes the Vrn-1H gene, but may coincide with the wheat 5A Rcg1 locus, which governs the threshold temperature at which cor genes are induced. From this, it remains possible that HvCbf3 is the basis of a minor quantitative trait locus in some genetic backgrounds, though that possibility remains to be thoroughly explored (**Dong *et al.*, 2002**)

The objectives of the present work is the following:

1. Isolation and cloning the stress tolerance gene(s) with emphases to salinity.
2. Sequencing and characterization of the isolated gene(s) *via* published genetic data base.
2. Study the effects of salt stress in barley leaves on gene expression profiling related to salt stress; DREB-CBF3-like gene using semi-quantitative RT-PCR.