

**DEVELOPMENT OF GENETICALLY MODIFIED  
STRAINS OF WHEAT EXPRESSING  
THE *HAL2*-LIKE GENE**

**By**

**SHEREEN ABU EL-MAATY MOHAMMED**

**B.Sc. Agric. Sci. (Biochemistry), Fac. Agric., Cairo Univ., Egypt, 1999**

**M.Sc. Agric. Sci. (Genetics), Cairo Univ., Egypt, 2005**

**THESIS**

**Submitted in Partial Fulfillment of the  
Requirements for the Degree of**

**DOCTOR OF PHILOSOPHY**

**In**

**Agriculture Sciences  
(Genetics)**

**Department of Genetics  
Faculty of Agriculture  
Cairo University  
EGYPT**

**2010**

APPROVAL SHEET

**DEVELOPMENT OF GENETICALLY MODIFIED  
STRAINS OF WHEAT EXPRESSING  
THE *HAL2*-LIKE GENE**

**Ph.D. Thesis  
In  
Agric. Sci. (Genetics)**

**By**

**SHEREEN ABU EL-MAATY MOHAMMED**  
B.Sc. Agric. Sci. (Biochemistry), Fac. Agric., Cairo Univ., Egypt, 1999  
M.Sc. Agric. Sci. (Genetics), Cairo Univ., Egypt, 2005

Approval Committee

**Dr. EFFAT ABD EL-LATIF BADR**.....  
Professor of Genetics, Fac. Agric., Alexandria University

**Dr. NAGLAA ABD EL-MONEEM ABDALLAH**.....  
Professor of Genetics, Fac. Agric., Cairo University

**Dr. EBTISSAM H. A. HUSSEIN** .....  
Professor of Genetics, Fac. Agric., Cairo University

**Dr. HASHEM AHMED HUSSEIN** .....  
Emeritus Professor of Genetics, Fac. Agric., Cairo University

**Date:**     /     / 2010

**SUPERVISION SHEET**

**DEVELOPMENT OF GENETICALLY MODIFIED  
STRAINS OF WHEAT EXPRESSING  
THE *HAL2*-LIKE GENE**

**Ph.D. Thesis  
In  
Agric. Sci. (Genetics)**

**By**

**SHEREEN ABU EL-MAATY MOHAMMED**  
B.Sc. Agric. Sci. (Biochemistry), Fac. Agric., Cairo Univ., Egypt, 1999  
M.Sc. Agric. Sci. (Genetics), Cairo Univ., Egypt, 2005

**SUPERVISION COMMITTEE**

**Dr. HASHEM AHMED HUSSEIN**  
Emeritus Professor of Genetics, Fac. Agric., Cairo University

**Dr. EBTISSAM HUSSEIN ALY HUSSEIN**  
Professor of Genetics, Fac. Agric., Cairo University

**Dr. AHMED BAHIELDIN MOHAMED**  
Professor of Genetics, Fac. Agric., Ain Shams University

**Name of Candidate:** Shereen Abu El-Maaty Mohammed      **Degree:** Ph.D.  
**Title of Thesis:** Development of Genetically Modified Strains of Wheat  
Expressing the *HAL2*-like Gene

**Supervisors:** Dr. Hashem Ahmed Hussein  
Dr. Ebtissam H.A. Hussein  
Dr. Ahmed Bahieldin Mohamed

**Department:** Genetics

**Approval:** 18/ 12/ 2010

### ABSTRACT

Wheat yield is severely affected by soil salinity. This study was conducted in an attempt to improving the salt stress tolerance of two wheat (*Triticum aestivum* L.) cultivars, i.e., Giza 164, an Egyptian wheat cultivar and Bobwhite 56. Immature embryo derived calli of the wheat genotypes were bombarded with a plasmid containing the rice halotolerance gene (*HAL2*-like gene) for salt stress tolerance and the *bar* gene for herbicide resistance. Three putatively transgenic plants from Bobwhite 56 cultivar namely B56-10, B56-20 and B56-24, and three putatively transgenic plants from Giza164 cultivar namely G164-5, G164-16 and G164-26 were detected by herbicide application through leaf painting. The transformation efficiency was 0.22 and 0.13 for Bobwhite 56 and G164, respectively. For molecular confirmation of putative transgenic plants, PCR analysis has been carried out and revealed the presence of both of the *HAL2*-like and the *bar* genes in the DNA of the putatively transgenic plants. Southern blot hybridization confirmed the integration of the gene of interest (*HAL2*-like) into the genome of the wheat transgenic plants. Moreover, RT-PCR analysis confirmed the expression of both of the *HAL2*-like and the *bar* genes. Sand culture experiment was conducted to investigate the effect of the introduced *HAL2*-like gene on wheat salt tolerance. T<sub>2</sub> transgenic plants and wild type plants were subjected to salt stress of 6000 ppm and 8000 ppm NaCl solution. The data showed that the transgenic lines expressed different levels of salt tolerance as expressed by the performance of the yield related traits. Transgenic lines G164-5/2 and B56-20/2 manifested the lowest reduction in yield traits performance. The results confirmed that the *bar* and the *HAL2*-like genes was successfully integrated into the genome of wheat, transmitted and expressed in the transgenic progeny.

**Key words:** wheat, immature embryos, *HAL2*-like gene, *bar* gene, salt stress, transformation, biolistic gun.

## DEDICATION

*I dedicate this work to whom my heart felt thanks: to soul of my Dear father, the most influential person in my life, who showed me what the attitude towards life's responsibilities should be., to my mother who brought me into this world and made me who I am with constant dedication and unconditional support, to my brothers and specially to my dear husband Taye and my dear children for their patience, support and encouragement they continually offered along the period of my post graduation.*

## **ACKNOWLEDGEMENT**

*At first, I would like to thank **ALLAH** that allowed me to achieve this work, without his bless any great effort is invaluable.*

*I wish to express my sincere thanks, deep gratitude and appreciation to **Dr. HASHEM A. HUSSEIN** Professor of Genetics, Faculty of Agriculture, Cairo University for suggesting the problem, supervision, continued assistance, guidance, great interest, encouragement, following the progress of the work with great interest and continuous criticism through the course of study.*

*My deepest and sincere graduate to **Dr. EBTISSAM H.A. HUSSEIN** Professor of Genetics, Faculty of Agriculture, Cairo University for suggesting the point of research, supervision, directing, continuous encouragement, continuous support for practical parts of this research work and constant writing the paper and the thesis.*

*I would like to express my appreciation to **Dr. AHMED BAHIELDIN MOHAMED**, Professor of Genetics, Faculty of Agriculture, Ain Shames University for supervision, planning, directing, following the progress of the work and great help during carrying out and preparation of this work,*

*I would like to express my deep appreciation to **Dr. HALA F. EISSA**, Senior Researcher of Environmental Stress Laboratory (ESL), Agricultural Genetic Engineering Research Institute (AGERI), for planning, directing, following the progress of the work and great help during carrying out and preparation of this work,*

*Many thanks are due to **Dr. AHMED M. RAMADAN** Researcher in Environmental Stress Laboratory (ESL), Agricultural Genetic Engineering Research Institute (AGERI), for his kind and continuous help and guidance during this work,*

*Many thanks are due to all the members of the Genetics Department, Faculty of Agriculture, Cairo University and all the members of Agricultural Genetic Engineering Research Institute (AGERI) for their continuous help, facilities and moral support.*

# CONTENTS

	Page
<b>INTRODUCTION</b> .....	1
<b>REVIEW OF LITERATURE</b> .....	4
<b>1. Effect of salinity on plants.</b> .....	4
<b>2. Plant transformation for abiotic stress tolerance</b> .....	14
<b>3. Wheat transformation</b> .....	21
a. Transformation of wheat for abiotic stress tolerance. ....	37
<b>4. Improvement of salinity stress tolerance through transfer of the halotolerance genes</b> .....	44
<b>MATERIALS AND METHODS</b> .....	53
<b>1. Materials</b> .....	53
a. Plant material .....	53
b. Plant expression vector .....	53
<b>2. Methods</b> .....	54
<b>a. Preparation of plasmid DNA</b> .....	54
1. Preparation of competent cells.....	54
2. Transformation of competent cells with plasmid DNA .....	56
3. Plasmid isolation.....	56
4. Plasmid construction confirmation.....	63
<b>b. Wheat transformation and regeneration</b> .....	64
1. Seed sterilization.....	64
2. Excision of immature embryos and callus initiation... ..	64
3. Osmotic treatment.....	65
4. Bombardment of immature embryo-derived callus... ..	66
5. Subculture of immature embryo-derived calli.....	69
6. Regeneration of putatively-transgenic plantlets.....	69
7. Acclimatization procedure.....	70
<b>c. Evaluation of putative transgenic plants</b> .....	70
1. Leaf painting with the herbicide Basta.....	71
2. Molecular analysis.....	71
<b>a. Molecular analysis at the structural level</b> .....	71
1. Genomic DNA extraction.....	71
2. Polymerase chain reaction (PCR) analysis.....	73

3. Genomic Southern analysis.....	75
<b>b. Molecular analysis at the functional level.....</b>	<b>81</b>
1. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis.....	81
<b>d. Evaluation of the herbicide resistance of T<sub>1</sub> and T<sub>2</sub> plants.....</b>	<b>85</b>
<b>e. Evaluation of the transgenic plants under salt- stress (green house experiment).....</b>	<b>85</b>
<b>f. Statistical analysis.....</b>	<b>86</b>
<b>RESULTS AND DISCUSSION.....</b>	<b>87</b>
<b>1. Verification of the recombinant plasmid harboring the HAL2-like gene and the <i>bar</i> genes.....</b>	<b>87</b>
<b>2. Wheat transformation .....</b>	<b>91</b>
a. Callus induction.....	91
b. Bombardment of immature embryo derived calli.....	92
c. Regeneration .....	96
d. Acclimatization .....	97
<b>3. Evaluation of putative transgenic plants.....</b>	<b>99</b>
a. Evaluation of putative transgenic plants with the herbicide Basta.....	99
<b>b. Molecular analysis.....</b>	<b>103</b>
1. Polymerase chain reaction (PCR) analysis.....	103
2. Genomic Southern blot analysis.....	106
3. Molecular analysis at the functional level.....	108
a. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis.....	108
<b>4. Evaluation of the transgenic plants under salt stress.....</b>	<b>110</b>
a. Evaluation of the G164-derived transgenic T <sub>2</sub> plants under salt stress.....	111
b. Evaluation of the B56-derived transgenic T <sub>2</sub> plants under salt stress.....	118
<b>SUMMARY .....</b>	<b>127</b>
<b>REFERENCES.....</b>	<b>131</b>
<b>ARABIC SUMMARY</b>	



## LIST OF TABLES

No	Title	Page
1.	Components of the TW medium used for callus induction.....	65
2.	Sequence of primers used in PCR analysis for detection of the <i>HAL2</i> -Like and the <i>bar</i> genes.....	73
3.	Dilution of Biotin-labeled control DNA for quantification of Biotin -labeled DNA.....	77
4.	Number of explants used for transformation, number of putatively transgenic plantlets and transformation frequency for the two wheat cultivars.....	98
5.	Mean performance of some yield-related traits of T <sub>2</sub> transgenic plants derived from G164 and the non-transgenic parent (G164) as affected by salt stress.....	112
6.	Percentage of reduction in the yield traits due to the salt treatments (6000 ppm and 8000 pp) for the non-transgenic (G164) and the transgenic lines.....	117
7.	Mean performance of some yield-related traits of T <sub>2</sub> transgenic plants derived from B56 and the non-transgenic parent (B56) as affected by salt stress.....	119
8.	Percentage of reduction in the yield traits due to the salt treatments (6000 ppm and 8000 pp) for the non-transgenic (B56) and the transgenic lines.....	123

## LIST OF FIGURES

No	Title	Page
1.	Schematic representation of the plasmid pHAL (7.32kb).....	54
2.	Probe yield concentrations of the <i>HAL2</i> -like gene fragment.....	78
3.	Agarose gel electrophoresis (1.2%) of pHAL plasmids (7.3 Kb) digested with <i>EcoRI</i> restriction enzyme. M, 1 Kb ladder. Lanes (1-5) recombinant plasmids.....	88
4.	PCR screening of the pHAL plasmid using specific primers to the coding sequence of the <i>bar</i> gene. M, 1 Kb ladder. Lane (1) recombinant plasmid.....	90
5.	Different stages for wheat transformation:(A) Immature embryos.(B) Calli on osmotic medium.(C) Callus growth after bombardment.(D) Shooted calli.(E) Rooted plantlets.(F) Regenerated wheat plant.....	94
6.	Leaf painting with the herbicide Basta: (A) Non-transformed plantlet. (B) Transformed plantlet of T <sub>0</sub> .....	100
7.	Evaluation of transgenic wheat plants in the field sprayed with 1 g/L Basta: (A) T <sub>1</sub> plants. (B) T <sub>2</sub> plants. (C) Non-transgenic plants (control).....	101
8.	PCR amplified DNA using the <i>bar</i> gene specific primers (A) and the <i>HAL2</i> -like gene specific primers (B). Lanes 1-2 represent T <sub>1</sub> transgenic progeny of G164-5, lanes 3-10 represent T <sub>1</sub> transgenic progeny of G164-16 and lanes 11-13 represent T <sub>1</sub> transgenic progeny of G164-26. Lane 14 represents T <sub>1</sub> transgenic progeny of B56-10, lanes 15-20 represent T <sub>1</sub> transgenic progeny of B56-24 and lanes 21-25	

represent T <sub>1</sub> transgenic progeny of B56-20. (M) 1 Kb DNA ladder. (+) positive control of pHAL. (C1) negative control of G164. (C2) negative control of B56.....	104
9. Genomic Southern analysis of some T <sub>1</sub> and T <sub>2</sub> transgenic plants of wheat. (1c & 5c) 1 and 5 copies of the <i>HAL2</i> -like gene; (G164) negative control of G164; (B56) negative control of Bobwhite 56. Lanes (1-3) represent transgenic plants of G164 (T <sub>1</sub> ), lanes 4-5 represent transgenic plants of G164 (T <sub>2</sub> ) and lanes (6-8) represent transgenic plants of B56 (T <sub>1</sub> ).....	107
10. RT-PCR product of partial-length of the <i>bar</i> gene (A) and the <i>HAL2</i> -like gene (B) from the T <sub>1</sub> transgenic plants. Lanes 1-2 represent T <sub>1</sub> transgenic progeny of G164-5, lanes 3-10 represent T <sub>1</sub> transgenic progeny of G164-16 and lanes 11-12 represent T <sub>1</sub> transgenic progeny of G164-26. Lane 13 represents T <sub>1</sub> transgenic progeny of B56-10, lanes 14-19 represent T <sub>1</sub> transgenic progeny of B56-24 and lanes 20-24 represent T <sub>1</sub> transgenic progeny of B56-20. (M) 1 Kb DNA ladder. (+) positive control of pHAL. (C1) negative control of G164. (C2) negative control of B56.....	109
11. Histogram illustrating means of some yield-related traits: (A) plant height; (B) flag leaf area; (C) spike length; (D) biomass/ plant and (E) grain yield/plant for the transgenic lines (G164-5/2, G164-26/15 and G164-16/9) and non-transgenic parent (G164) under salt stress.....	113
12. Histogram illustrating means of some yield-related traits: (A) plant height; (B) flag leaf area; (C) spike length; (D) biomass/ plant plant and (E) grain yield/plant for the transgenic lines (B56-10/1, B56-20/2 and B56-24/8) and non-transgenic parent (B56) under salt stress.....	120



## INTRODUCTION

Bread wheat (*Triticum aestivum* L.) is a hexaploid species ( $2n = 6x = 42$ , AABBDD) belonging to the family Poaceae.. Almost 95% of the wheat grown today is of the hexaploid type, while nearly all of the remaining 5% is durum wheat (tetraploid) (Patnaik and Khurana, 2001). It has a large genome of approximately 17000 Mb, a~8-fold larger than that of maize and a 40-fold larger than that of rice (Gill *et al.*, 2004). Wheat is a major staple food for more than one third of the world population, with an annual yield production of 616.8 million tons in 2006 ([http:// faostat.fao.org](http://faostat.fao.org)).

Abiotic stresses are the major environmental challenges for crops in most of the countries, they are considered as limiting factors of plant growth and crop yield. About 7% of the world's total land area is affected by salinity (Flowers *et al.*, 1997). Thus, salinity is one of the important factors responsible for low yield and restricted economic utilization of land resources both in arid and semi arid regions of the world. The 21 century is marked by global scarcity of water resources, environment pollution and increased salinization of soils and water (Tester and Bacic, 2005). Egypt is one of the countries that suffer severe salinity problems, as 33% of the cultivated land which represents only 3% of the total land area in Egypt is already salinized (Ghassemi *et al.*, 1995).

Wheat is known as moderately tolerant crop to salt. Nevertheless, the possibilities for increasing tolerance to abiotic stresses are enormous and genetic transformation is a powerful tool to develop salinity-tolerant genotypes. However, there are different

methods of transformation and different selection schemes. The most common approach for wheat transformation is the bombardment of tissue derived from immature embryos followed by selection based on resistance to the *bar* gene.

Many genes have been used for wheat transformation to improve salinity-stress tolerance such as: *HAL1* (Gaxiola *et al.*, 1992), *AKT1* (Sentenae *et al.*, 1992), *HAL2* (Glaser *et al.*, 1993), P5CSF129A (Zhang *et al.*, 1995), chimeric accumulating gene (*cpy/sacB*) (Eissa, 2001; Bahieldin *et al.*, 2003), *mtlD* (Abebe *et al.*, 2003; Ramadan, 2005), *HVA1* (Patnaik and Khurana, 2003; Bahieldin *et al.*, 2005), *LEA* (Brini *et al.*, 2007), antiporter *hvnhx2* (Miroshnichenko *et al.*, 2007), *ATHK1* (Chen *et al.*, 2009).

Even with all these successful reports, hurdles still exist for this recalcitrant crop. Of these hurdles, low transformation rates, tools for transgene expression, and transgene silencing in subsequent generations are probably the most critical (Weeks *et al.*, 1993; Becker *et al.*, 1994; Janakiraman *et al.*, 2002; Sahrawat *et al.*, 2003; Huw *et al.*, 2005).

The rice halotolerance gene (*HAL2*-like gene) or RHL gene belongs to the inositol monophosphatase family. This gene encodes a cation-sensitive nucleotidase required for sulfate assimilation and RNA processing. The *HAL2* gene was identified in yeast by functional assay for supporting the growth of cells under high salinity stress (Glaser *et al.*, 1993) and over expression of this gene in yeast improved salt tolerance. Peng and Verma (1995) isolated and characterized rice *HAL*-like (RHL) cDNA. The RHL cDNA complemented both *cysQ*

and met 22 mutants in bacteria and yeast, respectively. Thus, they have demonstrated that the proteins encoded by *cysQ*, *HAL2* and *RHL* genes have the same function in sulfur assimilation pathway in *E.coli*, yeast and plants. They have further shown that the enzyme encoded by the *RHL* gene is 3'(2'), 5'-diphosphonucleoside and uses both adenosine 3'-phosphate 5'-phosphosulfate (PAPS) and 3'(2')-phosphoadenosine 5'-phosphate (PAP) as substrates. Availability of active sulfur seems essential to overcome oxidative stress imposed by salinity and drought stresses. Over expression of this gene in plants resulted in accumulation of glutathione which also reduces oxidative stress.

**Thus, the present investigation was carried out to address the following objectives:**

1. To transform an Egyptian wheat cultivar (Giza 164) and an American wheat cultivar (Bobwhite 56) using the biolistic particle delivery system and the plasmid pHAL harboring the *HAL2*-like gene for salt tolerance beside the *bar* gene as a selectable marker.
2. To produce putative transgenic wheat plants tolerant to salt stress.
3. To confirm the expression of the *bar* gene by leaf painting and herbicide spraying.
4. To confirm the integration of the introduced genes in the putative transgenic plants using PCR and Southern blot analyses and the expression using RT-PCR.
5. To evaluate the performance of transgenic wheat lines constitutively expressing the *HAL2*-like gene based on yield and yield-related traits under saline as well as non saline conditions.