

**GENETIC ENGINEERING APPROACHES TO  
IMPROVE ABIOTIC STRESS RESISTANCE IN  
EGYPTIAN WHEAT**

**BY**  
**AHMED MOHAMMED MOHAMMED RAMADAN**  
B.Sc. Agric. Sc. (Genetics), Ain Shams University, 1999

**A thesis submitted in partial fulfillment  
of  
the requirements for the degree of**

**MASTER OF SCIENCE**  
**in**  
**Agricultural Science**  
**(Genetics)**

**Department of Genetics**  
**Faculty of Agriculture -Ain Sham University**

**2005**

# **GENETIC ENGINEERING APPROACHES TO IMPROVE ABIOTIC STRESS RESISTANCE IN EGYPTIAN WHEAT**

**BY**

**AHMED MOHAMMED MOHAMMED RAMADAN**

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

**Under the supervision of:**

**Prof. Dr. Ahmed Bahieldin Mohamed**

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

**Prof. Dr. Fotouh M. El-Domyati**

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

**Dr. Hesham Taha Mahfouz**

Senior researcher of Genetics, Agricultural Genetic  
Engineering Research Institute (AGERI), Agricultural  
Research Center (ARC)

Approval sheet

**GENETIC ENGINEERING APPROACHES TO  
IMPROVE ABIOTIC STRESS RESISTANCE IN  
EGYPTIAN WHEAT**

**BY**

**AHMED MOHAMMED MOHAMMED RAMADAN**

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

**This thesis for M. Sc. degree has been approved by:**

**Prof. Dr. Naglaa Abd El Moneim Abdallah** .....  
Prof. of Genetics, Faculty of Agriculture, Cairo University

**Prof. Dr. Fatthy Mohamed Abdel-Tawab** .....  
Prof. Emeritus of Genetics, Faculty of Agriculture, Ain Shams  
University

**Prof. Dr. Fotouh Mohamed El-Domyati** .....  
Prof. of Genetics, Faculty of Agriculture, Ain Shams  
University

**Prof. Dr. Ahmed Bahieldin Mohamed** .....  
Prof. of Genetics, Faculty of Agriculture, Ain Shams  
University

Date of Examination     /     / 2005

## ACKNOWLEDGMENT

**First and foremost, I feel always indebted to  
Allah, the most beneficent and merciful**

I wish to express my deep thanks and gratitude to Prof. Dr. **Ahmed Bahieldin**, Professor of Molecular Genetics, Department of Genetics, Faculty of Agriculture, Ain Shams University for his supervision, guidance, and every possible help he kindly offered during the course of this investigation.

I also like to express my deepest gratitude and sincere appreciation to Prof. Dr. **F. M. El-Domyati**, Professor of Molecular Genetics, Faculty of Agriculture, Ain Shams University for his continuous supervision, kind help and valuable comments through the course of this study.

I would like to thank Dr. **Hesham Mahfouz**, for his practical help and continuous encouragement throughout the course of this study.

I would like to express my most sincere gratitude to Prof. Dr. **Hanaiya El-Itriby**, Director of the Agricultural Genetic Engineering Research Institute for her kind support and encouragement.

I would like to express my deep thanks to. Dr. **Hala Eissa**, Senior Scientist at Agricultural Genetic Engineering Research Institute and Dr. **Osama Saleh**, Senior Scientist at National Center of Radiation Research and Technology, Egypt, For thier energetic guidance and conclusive instructions and practical help throughout the course of this study.

I am very grateful to my colleagues at the ESL, **Sherif Edris, Hoda El-Garhi, Sameh El-Sayed, Omnia Osama, Sara Khairy and Adel Abdurrady** for their continuous encouragement and help in the preparation of this work. Also, I would like to extend my thanks to all the AGERI stuff, who have helped me. I must especially acknowledge **Ahmed Shokry** and **Walid Moneer** for their continuous support and help.

Also, I would like to thank all the stuff at Department of Genetics Faculty of Agriculture, Ain Shams University especially Molecular

Cytogenetic Lab, I must especially acknowledge **Prof. Dr. El-Sayed H. Hassanien**, Professor of Genetics, Dept. of Genetics, Fac. of Agric., Ain Shams Univ. for their continuous encouragement and help.

Finally, I would like to thank my family, my father, mother and my brothers for their encouragement and support during this work.

## ABSTRACT

**Ahmed Mohammed Mohammed Ramadan, Genetic engineering approaches to improve abiotic stress resistance in Egyptian wheat Unpublished master of Science Thesis, Genetics Dept., Fac. Agric., Ain Shams Univ., 2005.**

The bacterial gene, *mtlD*, that encodes mannitol-1-phosphate dehydrogenase, for mannitol accumulation was introduced into Egyptian bread wheat cv. Giza 163. The transformation was carried out by particle bombardment of immature embryos using the biolistic device. A plant expression vector was constructed in which *mtlD* gene was driven by the *ubi* promoter, and the selectable marker *bar* encoding the phosphinothricin acetyl transferase gene for herbicide resistance was driven by the *CaMV* 35S promoter. Two T<sub>0</sub> plants were regenerated and submitted to preliminary evaluation by leaf painting with the herbicide basta (1 g/L) and showed no symptoms of necrosis. The two putatively transgenic plants were submitted to PCR and Southern analysis, in which the latter showed that one copy of the *mtlD* gene has been incorporated. Subsequent molecular testing was carried out to determine gene expression in transformed T<sub>1</sub> wheat plants using northern analysis. Salt stress experiment in sand culture indicated that one of the two transgenic events could tolerate intermediate concentration of salt (8000 ppm 3 NaCl/1 CaCl<sub>2</sub>). Under salt stress, flag leaf area in transgenic plants was reduced by 60%, plant height by 35%, peduncle length by 24%, 1000 grain weight by 26%, biological yield by 74%, and grain yield by 40%. In wild type, flag leaf area was reduced by 53%, plant height by 40%, peduncle length by 46%, 1000-grain weight by 58%, biological yield by 71%, and grain yield by 66%. Finally, we concluded that this approach can be used in improving abiotic stress tolerance in wheat

**Key words:** Wheat, immature embryos, transformation, biolistic bombardment, drought tolerance, Mannitol, leaf-painting, bialaphos, PCR, genomic Southern, Northern.

# إستخدام طرق الهندسة الوراثية لتحسين المقاومة للضغوط غير الحيوية فى القمح المصرى

رسالة مقدمة من

أحمد محمد محمد رمضان

بكالوريوس علوم زراعية (وراثة) ، جامعة عين شمس، 1999.

للحصول على  
درجة الماجستير فى العلوم الزراعية  
(وراثة)

قسم الوراثة  
كلية الزراعة  
جامعة عين شمس

2005

صفحة الموافقة على الرسالة

# إستخدام طرق الهندسة الوراثية لتحسين المقاومة للضغوط غير الحيوية فى القمح المصرى

رسالة مقدمة من

أحمد محمد محمد رمضان

بكالوريوس علوم زراعية (وراثة) ، جامعة عين شمس، 1999.

للحصول على  
درجة الماجستير فى العلوم الزراعية  
(وراثة)

وقد تمت مناقشة الرسالة والموافقة عليها

اللجنة

أ.د.

أ.د.

أ.د.

تاريخ المناقشة :

لجنة الإشراف

# إستخدام طرق الهندسة الوراثية لتحسين المقاومة للضغوط غير الحيوية فى القمح المصرى

رسالة مقدمة من

أحمد محمد محمد رمضان

بكالوريوس علوم زراعية (وراثة) ، جامعة عين شمس، 1999.

للحصول على  
درجة الماجستير فى العلوم الزراعية  
(وراثة)

تحت إشراف

أ.د./ أحمد بهى الدين

أستاذ الوراثة الجزيئية - قسم الوراثة  
كلية الزراعة - جامعة عين شمس

أ.د./ فتوح محمد عبد المجيد الدمياطى

أستاذ الوراثة الجزيئية - قسم الوراثة  
كلية الزراعة - جامعة عين شمس

أ.د./ هشام أحمد طه محفوظ

باحث أول - معهد بحوث الهندسة الوراثية الزراعية  
- مركز البحوث الزراعية

جامعة عين شمس

كلية الزراعة

رسالة ماجستير

اسم الطالب: أحمد محمد محمد رمضان

عنوان الرسالة: إستخدام طرق الهندسة الوراثية لتحسين المقاومة للضغط غير

الحيوية في القمح المصرى

لجنة الإشراف:

أ.د./ أحمد بهى الدين

أستاذ الوراثة الجزيئية - قسم الوراثة

كلية الزراعة - جامعة عين شمس (المشرف الرئيسى)

أ.د./ فتوح محمد عبد المجيد الدمياطى

أستاذ الوراثة الجزيئية - قسم الوراثة

كلية الزراعة - جامعة عين شمس

أ.د./ هشام أحمد طه محفوظ

باحث أول - معهد بحوث الهندسة الوراثية الزراعية

- مركز البحوث الزراعية

تاريخ البحث : / / 2005

الدراسات العليا

أجيزت الرسالة بتاريخ : / /

موافقة مجلس الجامعة

/ /

ختم الإجازة

موافقة مجلس الكلية

/ /

## TABLE OF CONTENTS

|                                                                                                  |                                     |
|--------------------------------------------------------------------------------------------------|-------------------------------------|
| <b>I. INTRODUCTION .....</b>                                                                     | <b>ERROR! BOOKMARK NOT DEFINED.</b> |
| <b>II. REVIEW OF LITERATURE ...</b>                                                              | <b>ERROR! BOOKMARK NOT DEFINED.</b> |
| 1. Improvement of abiotic stress tolerance through genetic transformation.....                   | <b>Error! Bookmark not defined.</b> |
| 1.1. Transfer of Mannitol-accumulating gene ( <i>mtlD</i> ) using genetic engineering approaches | <b>Error! Bookmark not defined.</b> |
| 1.2. Other genes for abiotic stress tolerance                                                    | <b>Error! Bookmark not defined.</b> |
| 2. Wheat transformation .....                                                                    | <b>Error! Bookmark not defined.</b> |
| 2.1. Establishment of transformation protocols                                                   | <b>Error! Bookmark not defined.</b> |
| <b>III. MATERIALS AND METHOD</b>                                                                 | <b>ERROR! BOOKMARK NOT DEFINED.</b> |
| 1. Materials .....                                                                               | <b>Error! Bookmark not defined.</b> |
| 1.1. Plant material .....                                                                        | <b>Error! Bookmark not defined.</b> |
| 1.2. Plant expression vector.....                                                                | <b>Error! Bookmark not defined.</b> |
| 2. Methods .....                                                                                 | <b>Error! Bookmark not defined.</b> |
| 2.1. Gene construction.....                                                                      | <b>Error! Bookmark not defined.</b> |
| 2.1.1. Enzymatic digestion.....                                                                  | <b>Error! Bookmark not defined.</b> |
| 2.1.2. Fill up of sticky ends .....                                                              | <b>Error! Bookmark not defined.</b> |
| 2.1.3. Ligation .....                                                                            | <b>Error! Bookmark not defined.</b> |
| 2.1.4. Introduction of plasmid DNA into <i>E. coli</i> competent cells.....                      | <b>Error! Bookmark not defined.</b> |
| 2.1.4.1. Preparation of competent cells                                                          | <b>Error! Bookmark not defined.</b> |
| 2.1.4.2. Transformation of competent cells with plasmid DNA .....                                | <b>Error! Bookmark not defined.</b> |
| 2.1.5. Plasmid isolation and confirmation of construction                                        | <b>Error! Bookmark not defined.</b> |
| 2.1.5.1. Minipreparation of the plasmid                                                          | <b>Error! Bookmark not defined.</b> |
| 2.1.5.2. Maxi preparation of the plasmid construct .....                                         | <b>Error! Bookmark not defined.</b> |
| 2.1.5.3 Plasmid confirmation....                                                                 | <b>Error! Bookmark not defined.</b> |

|                                                                       |               |                              |
|-----------------------------------------------------------------------|---------------|------------------------------|
| 2.1.5.4. Determination of DNA concentration                           | Error!        | Bookmark not defined.        |
| 2.1.5.5. Agarose gel electrophoresis                                  | Error!        | Bookmark not defined.        |
| 2.2. Wheat transformation and regeneration                            | Error!        | Bookmark not defined.        |
| 2.2.1. Surface sterilization of seeds                                 | Error!        | Bookmark not defined.        |
| 2.2.2. Excision of immature embryos and callus Initiation             | Error!        | Bookmark not defined.        |
| 2.2.3. Osmotic treatment .....                                        | Error!        | Bookmark not defined.        |
| 2.2.4. Bombardment of immature embryo-derived callus                  | Error!        | Bookmark not defined.        |
| 2.2.4.1. Preparation of microcarriers for bombardment ..              | Error!        | Bookmark not defined.        |
| 2.2.4.2. The gun conditions.....                                      | Error!        | Bookmark not defined.        |
| 2.2.4.3. Operation of the biolistic gun                               | Error!        | Bookmark not defined.        |
| 2.2.4.4. Transformation experiments                                   | Error!        | Bookmark not defined.        |
| 2.2.5. Subculture of immature embryo-derived callus                   | Error!        | Bookmark not defined.        |
| 2.2.6. Production of regenerated putatively-transgenic explants. .... | Error!        | Bookmark not defined.        |
| 2.2.7. Acclimatization procedure                                      | Error!        | Bookmark not defined.        |
| 2.3. Evaluation of putative transgenic plants                         | Error!        | Bookmark not defined.        |
| 2.3.1. Leaf painting with the herbicide basta                         | Error!        | Bookmark not defined.        |
| 2.3.2. Molecular analysis .....                                       | Error!        | Bookmark not defined.        |
| 2.3.2.1. Molecular analysis on the structural level.....              | Error!        | Bookmark not defined.        |
| 2.3.2.2. Molecular analysis at the functional level.....              | Error!        | Bookmark not defined.        |
| 2.4. Greenhouse experiment .....                                      | Error!        | Bookmark not defined.        |
| <b>IV. RESULTS AND DISCUSSION</b>                                     | <b>Error!</b> | <b>BOOKMARK NOT DEFINED.</b> |
| 1. Construction of plant expression vector.                           | Error!        | Bookmark not defined.        |
| 2. Wheat tissue culture and transformation                            | Error!        | Bookmark not defined.        |
| 2.1. Callus induction .....                                           | Error!        | Bookmark not defined.        |
| 2.2. Regeneration.....                                                | Error!        | Bookmark not defined.        |

|                                                        |                              |
|--------------------------------------------------------|------------------------------|
| 2.3. Callus rooting and plantlet acclimatization       | Error! Bookmark not defined. |
| 3. Evaluation of putative transgenic plants            | Error! Bookmark not defined. |
| 2.3.1. Leaf painting with the herbicide basta          | Error! Bookmark not defined. |
| 3.2. Molecular analysis of transgene(s)                | Error! Bookmark not defined. |
| 3.2.1. On the structural level .....                   | Error! Bookmark not defined. |
| 3.2.2. On the expression level .....                   | Error! Bookmark not defined. |
| 3.3. Evaluation of transgenic plants under salt stress | Error! Bookmark not defined. |
| V. SUMMARY .....                                       | ERROR! BOOKMARK NOT DEFINED. |
| VI. REFERENCES .....                                   | ERROR! BOOKMARK NOT DEFINED. |
| ARABIC SUMMARY                                         |                              |

## List of abbreviations

|               |                                                                |
|---------------|----------------------------------------------------------------|
| AP1           | Proteinase inhibitor                                           |
| Bip           | Endoplasmic reticulum response binding protein                 |
| Camv          | Cauliflower mosaic virus                                       |
| CMO           | Choline mono oxygenase                                         |
| DEX           | Dexamethasone                                                  |
| DON           | Deoxynivalenol                                                 |
| DRE           | Dehydration response element                                   |
| EPSPs         | 5-enolpyruvylshikimate-3-phosphate synthase                    |
| Fad7          | Omega-3-fatty acid desaturase                                  |
| FHB           | Fusarium head blight                                           |
| G163          | Giza163                                                        |
| GPD           | Glycerol-3-phosphate dehydrogenase                             |
| GUS gene      | $\beta$ -glucourindase                                         |
| HMW           | High molecular weight glutenin                                 |
| hpt           | Hygromycin phosphor transferase                                |
| HVA1          | One of a late embryogenesis abundant (LEA) protein genes       |
| KP4           | Antifungal protein from <i>Ustilago maydis</i> infection virus |
| LMW           | Low molecular weight glutenin                                  |
| MAPKKK        | Mitogene activated protein kinase kinase kinase                |
| MS media      | Murashige and skoog media                                      |
| mtID          | Mannitol-1-phosphate dehydrogenase                             |
| NMR           | Nuclear magnetic resonance                                     |
| nptII         | Kanamycin resistance gene                                      |
| osCDPK        | Calcium-dependent protein kinase                               |
| OSML          | Osmotin-like protein genes                                     |
| otsA and otsB | Trehalose biosynthetic genes                                   |
| PPT           | Phosphinothircin                                               |
| PR            | Pathogenesis related protein                                   |
| P-ubi         | Ubiquitin promoter                                             |
| RM1           | Cytokinin containing media                                     |
| SA            | Salicylic acid                                                 |
| SacB          | Fructan accumulation gene                                      |
| SOD           | Super oxide dismutase                                          |
| WSMV          | Wheat streak mosaic virus                                      |
| WUE           | Water use efficiency                                           |

**LIST OF TABLES**

| No.                                                                                                                                        | Page |
|--------------------------------------------------------------------------------------------------------------------------------------------|------|
| 1. Codon alignment of the bacterial <i>mtlD</i> gene with selected wheat genes as well as the bacterial <i>bar</i> gene (as a refree)..... | 65   |
| 2. Yield and yield attributes of G163 and transgenic plant 79 under different environmental conditions. ....                               | 84   |