

PRODUCTION OF CUCURBIT PLANTS EXPRESSING AN ANTIFUNGAL GENE

By

HOSSAM MOHAMED ZAKARIA

B.Sc. Agric. Sci. (Biochemistry), Fac. Agric., Cairo Univ. Egypt, ٢٠٠٠

THESIS

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

MASTER OF SCIENCE

In

**AGRICULTURAL SCIENCES
(Genetics)**

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

٢٠٠٧

APPROVAL SHEET

PRODUCTION OF CUCURBIT PLANTS EXPRESSING AN ANTIFUNGAL GENE

M.sc. Thesis
By

HOSSAM MOHAMED ZAKARIA

B.Sc. Agric. Sci. (Biochemistry), Fac. Agric., Cairo Univ. Egypt, ٢٠٠٠

Approved by:

Dr. MAHMOUD M. SAKER
Professor of Plant Biotechnology, National Research Center.

Dr. MONA H. HESSIN
Professor of Genetics, Fac. Agric., Cairo University.

Dr. NAGLAA A. ABDALLAH
Professor of Genetics, Fac. Agric., Cairo University.

Date: / /

SUPERVISION SHEET

**PRODUCTION OF CUCURBIT PLANTS
EXPRESSING AN ANTIFUNGAL GENE**

M.sc. Thesis
By

HOSSAM MOHAMED ZAKARIA
B.Sc. Agric. Sci. (Biochemistry), Fac. Agric., Cairo Univ. Egypt, ٢٠٠٠

SUPERVISION COMMITTEE

Dr. NAGLAA A. ABDALLAH
Professor of Genetics, Fac. Agric., Cairo University

Dr. ATEF S. SADIK
Professor of Microbiology, Fac. Agric., Ain Shams University

CONTENTS

	Page
INTRODUCTION.....	1
REVIEW OF LITERATURE	5
1. Fusarium wilt of watermelon.....	6
2. Plant regeneration	7
3. Cucurbits regeneration.....	8
4. Watermelon regeneration.....	12
5. Watermelon transformation.....	14
a. Transformation via <i>Agrobacterium</i> -mediated method.....	14
b. Transformation via biolistic gun method.....	15
6. Production of transgenic watermelon plants.....	16
7. Production of genetically modified plants against fungal diseases using the defensin gene.....	17
MATERIALS AND METHODS.....	21
1. MATERIALS.....	21
a. Plant materials.....	21
b. Tissue culture chemicals.....	21
c. Plasmids.....	21
d. Bacterial strains.....	23
e. Primers.....	23
2. METHODS.....	24
a. Regeneration conditions.....	25
b. Establishment of watermelon regeneration system.....	25
1. Explants preparation.....	25
2. Shoot regeneration.....	25
3. Shoot elongation.....	26
4. Root formation and acclimatization.....	27
c. Watermelon transformation.....	27
1. <i>Agrobacterium</i> -mediated transformation system.....	27

۲. Microprojectile bombardment transformation system.....	۲۸
a. Preparation of plasmid DNA for transformation.....	۲۸
b. Sterilization of gold particles and coating with DNA.....	۲۹
c. Bombardment and regeneration of bombarded explants.....	۳۰
d. Transformation with the <i>Msdef I</i> gene.....	۳۰
e. Evaluation of the transgenic watermelon plants.....	۳۱
a. Gus histochemical assay.....	۳۱
b. Leaf painting	۳۱
c. Polymerase chain reaction.....	۳۱
۱. DNA extraction.....	۳۱
۲. PCR assay.....	۳۳
d. Southern blot analysis	۳۳
e. Digestion of the plant genome DNA	۳۳
f. Southern blot and hybridization.....	۳۳
RESULTS AND DISCUSSION.....	۳۵
۱. Watermelon regeneration.....	۳۵
a. Effect of carbon source on watermelon regeneration.....	۳۹
b. Elongation and root formation stages.....	۴۰
۲. Establishment of the watermelon transformation	۴۲
a. <i>Agrobacterium</i> transformation of watermelon.....	۴۲
۱. Expression of <i>gus</i> gene	۴۳
۲. Expression of <i>bar</i> gene	۴۴
۳. PCR detection of transgenic plants.....	۴۵
b. Microprojectiel transformation of watermelon.....	۴۶
۱. Expression of <i>gus</i> gene.....	۴۸
۲. Expression of <i>bar</i> gene.....	۵۰
۳. PCR detection of transgenic plants.....	۵۱
۳. Watermelon transformation using the <i>Msdef I</i> gene.....	۵۲
۴. Evaluation of the putative transgenic plants for <i>Msdef</i> gene	۵۴
a. PCR assay.....	۵۴
b. Southern blot hybridization analysis.....	۵۵
Conclusions.....	۵۷
SUMMARY.....	۵۸

REFERENCES.....	٦٢
ARABIC SUMMARY.....	

LIST OF FIGURES

No	Title	Page
1.	Diagram showing the plant expression genes between the two boards of the binary vector pISV ²⁶⁷⁸	22
2.	Diagram showing the plasmid pAB ⁷	22
3.	Diagram showing the pMON ²⁶⁶³ plasmid containing the <i>MsDeFI</i> exon gene under the control of 30S promoter and nos terminator.....	23
4.	Regeneration stages of watermelon using the proximal zone of hypocotyls.....	38
5.	GUS histochemical assay of transformed hypocotyl explant treated with the <i>Agrobacterium</i> strain LBA ⁴³⁴ carrying the plasmid pISV ²⁶⁷⁸	44
6.	Leaf painting analysis of melon leaves showing necrosis on the non- transgenic leaf (left) and normal green color on the bialaphos-resistant leaf (right).....	45
7.	PCR analysis of putative <i>Agrobacterium</i> -transformed watermelon plants (Lanes 1-19) using <i>gus</i> -specific primers to amplify 2070 bp. Amplicons of the full <i>gus</i> -intron gene were presented in 7 out of the 19 tested plants. M: 100 bp DNA ladder plus.....	47
8.	PCR analysis of DNA from putative <i>Agrobacterium</i> -transformed watermelon (Lanes, 1-19) using <i>bar</i> -specific primers to amplify 540 bp fragment of the gene. Only seven out of 19 gave a positive amplicons. M: 100 bp DNA ladder.	47
9.	GUS histochemical assay of bombarded hypocotyl explant. Transformed explant gave blue dots (left), while the non-transformed explants remained colorless (right)	49
10.	PCR analysis for transformed plants bombarded with pAB ⁷ using <i>gus</i> gene specific primers to amplify a fragment of 1800 bp.....	51
11.	PCR analysis of DNA from watermelon plants (Lanes, 1-19) bombarded with pAB ⁷ plasmid, using <i>bar</i> -specific primers to amplify 540 bp of the gene. Only seven out of 19 gave a positive amplicons. M: 100 bp DNA	52

ladder.....	
۱۲. PCR analysis of putative watermelon plants (Lanes ۱-۱۹) bombarded with the plasmid pMON۲۲۶۵۳, amplifying ۲۵۰ bp. M: ۱۰۰ bp DNA ladder.....	۵۵
۱۳. Southern blot hybridization for the <i>Hind</i> III digested genome for two transgenic watermelon plants (lanes ۲ and ۳) and non –transgenic plant (lane ۱) as a negative control.....	۵۶

LISTT OF TABLES

No	Title	Page
١.	The nucleotide sequences of the primers used for PCR analysis.....	٢٤
٢.	Different shoot formation media compostion.....	٢٥
٣.	Different shoot elongation media compostion.....	٢٦
٤.	Watermelon regeneration frequency on different media using the proximal zone of hypocotyls explant.....	٣٦
٥.	Carbon source effect on the regeneration of watermelon cv. Giza\.....	٤٠
٦.	Effect of different media composition on the shoot elongation and on the percentage of vertification in the watermelon plants.....	٤١
٧.	Histochemical GUS assay of bombarded watermelon explants cvs. Giza \ and Giza ٢\.....	٤٩
٨.	Effect of different DNA concentrations and pressures on transformation frequency of watermelon cultivars Giza \ Giza ٢\ using pAB٦ plasmid and hypocotyl explant.....	٥٠

Name of Candidate: Hossam Mohamed Zakaria **Degree:** MS.C.
Title of Thesis: Production of cucurbit plants expressing an antifungal gene.
Supervisors: Prof. Dr. Naglaa A. Abdelallah and Prof. Dr. Atef S. Sadik,
Department: Genetics
Branch: Genetics **Approval:** / /

ABSTRACT

Watermelon is among the most important vegetable crops of the *Cucurbitaceae*. Members of this family are susceptible to different kinds of diseases such as fungal infection. In this investigation, an attempt to establish an efficient regeneration and transformation systems of the Egyptian watermelon cvs Giza 1, Giza 2 to produce transgenic plants resistant for fungal infection have been established. The proximal zone of hypocotyls was used as explants with four shoot formation media containing different concentrations of BAP, AgNO₃ and ABA. The regeneration system of the watermelon cultivars Giza 1 and Giza 2 was improved by adding 1 mg/l BA, 0.2 mg/l ABA and 0 mg/l AgNO₃ (Media WM₁) as it gave the highest respond of hypocotyl explants. The enhanced shoots were evaluated for elongation stage on four different elongation media. EWM₁ medium, containing 0.05 µg/l BAP, was considered as the best medium (82% of shoots were elongated with only 1% vitrification). The elongated shoots produced roots on MS supplemented with NAA at concentration of 0.5 µg/l for both Giza 1 (88,8%) and Giza 2 (79%). Transformation of explants was established using DNA bombardment with the plasmid pAB₁ carrying *gus* and *bar* genes as well as with *Agrobacterium*-mediated transformation method with the binary vector pISV₁ harboring the *gus*-intron and *bar* genes. Integration of the transgenes in the genome of putative transgenic plants and expression of transgenes was verified via histochemical assay, PCR and leaf painting tests. Furthermore, the plasmid pMON₁ containing the defensin gene, isolated from the leaves of alfalfa seedlings, under the control of the 35S promoter was introduced into the cultivar Giza 1 via biolistic gene transfer to develop resistance against the fungal infection. Putative transformants plants were screened using PCR and Southern blot analysis. Total genomic DNA of transformed plants were digested with *Hind*III and hybridized with labeled *Msdef1* gene. Positive labeled bands were obtained at different size fragments indicating that the gene allocated in different position in their genomes.

Keywords: Proximal zone of hypocotyls, *Agrobacterium*-mediated transformation, DNA bombardment, vitrification, defensin gene

ACKNOWLEDGMENT

I would like to express my sincere gratitude to Prof. Dr. Naglaa A. Abdallah, Prof. of Genetics, Genetics Department, Faculty of Agriculture, Cairo University for her supervision, continous guidance, and fruitful discussion.

Thanks are also due to Prof. Dr. Atef S. Sadik, Prof. of Agric. Microbiol., Department of Agric. Microbiol., Faculty of Agriculture, Ain Shams University and Dr. Gihan M. Hosny, Senior Scientist of PGTL, AGERI, ARC, Giza for their sincere help and supervision during the accomplishment of this manuscript.

Thanks also due to Prof. Dr. Hanaiya A. El-Itriby, former President of ARC for her endless help, encouragement during this work.

I would like to express my thanks to Dr. Roba M. Ismail and my colleagues; El-Dessoky, Attia, Ismail, Tarek and Khaled Hashem at AGERI, ARC, for their encouragement during this study.

INTRODUCTION

Cucurbitaceae is an economically important family which includes 118 genera with 820 species, and is widely distributed throughout tropical and subtropical regions (Whitaker and Davis, 1962). In Egypt, the cultivated area of melon is 40,000 hectares, providing an export value that exceeds 300,000 dollars annually (FAOSTAT, 2000). Plant diseases caused by fungi are often one of the most destructive agent and difficult to control. Watermelon is infected with a large number of fungal diseases including *Fusarium*, downy mildew, powdery mildew, etc. Serious diseases caused by fungi are usually controlled by chemical fungicides that the farmer applies to crops. Two different types of fusarium infection affect watermelon cultivation all over the world. Fusarium crown and foot rot is caused by *Fusarium solani* f. sp. *Cucurbitae* which exhibits host specificity for all cucurbits. Also, Fusarium wilt is caused by a seed- and soilborne fungus that is specific to melon and watermelon (Bruton *et al.* 2007).

Genetic engineering has opened new avenues to modify crops, and provided new solutions to solve specific needs. The powerful combination of genetic engineering and conventional breeding programs allows useful traits to be introduced into commercial crop varieties within an economically appealing time frame (Hansen and Wright, 1999).

Plants are amazingly adaptive at meeting environmental stresses and attacks from insects, fungi and viruses. Over the millennia and throughout millions of generations, plants have developed innovative

ways to survive and prosper under harsh conditions. Several molecules in plants have been reported to contribute to plant defense activity: proteins or peptides, such as lectins, ribosomal inactivating proteins, chitinases, proteases, defensins, peroxidases, ubiquitin-like peptides, ribonucleases, arginine- and glutamate-rich peptides and some unclassified proteins, organic compounds, classified into phytoanticipins and phytoalexins, which include phenols and phenolic glycosides, unsaturated lactones, sulphur compounds, saponins and dienes and active nitrogen and oxygen species, such as reactive nitrogen oxide species and hydrogen peroxide (Wang and Ng, 2002).

Higher plants have developed a range of systems by which to protect themselves from damage associated with biotic and abiotic stress (Tregear *et al.*, 2002).

Plants have developed a first line of defense toward off attacking fungi, called defensins. Plant defensins facilitate genetic modification of crops for enhanced resistance to fungal attacks. In general, plant defensin blocks calcium channels, which are used as a communication network by the fungus to direct its spread by disrupting the calcium pathway that will inhibit fungi growth. A number of studies has been performed to unravel the mode of action of plant defensins. It has been shown that plant defensins induce an array of relatively rapid responses in fungal membranes, including increased Ca^{2+} uptake, K^{+} efflux, increased uptake of fluorescent dyes, and membrane potential changes. Furthermore, the existence of high-affinity binding sites for plant defensins on fungal cells and plasma membrane fractions has been demonstrated (Thevissen *et al.*, 2000). The first step in the path

leading to fungal growth inhibition would be the binding of plant defensins to specific sites on the plasma membrane of fungal hyphae. Interaction with these binding sites would subsequently enable plant defensins to insert into the plasma membrane, thus affecting membrane structure and permeability to certain solutes, such as Ca^{2+} and K^+ , some of which play an important role in fungal growth and development.

In vitro regeneration of adventitious shoots is a critical stage of genetic transformation as they increase the frequency of transformation. Recently, a novel direct shoot regeneration using the proximal hypocotyl was efficiently applied in melon by Curuk *et al.* (2002), flax by Dedicova *et al.* (2003), cotton by Ouma *et al.* (2004) and canola by Cardoza and Stewart (2003).

Fungal diseases are the one of the most important problems that restrict the production of watermelon. Genetic engineering approach is a valuable biotechnology for breeding purposes and can be used to introduce resistant gene not found in the gene pool of particular species.

The main aim of this work was to produce transgenic watermelon plants resistant to fungal infection, especially fusarium using the plant defensin gene. Achieving this goal required the following:

1- To improve the previously developed regeneration system of the two Egyptian watermelon cultivars Giza1 and Giza2 using the proximal zone of hypocotyls.

٢- To compare between *Agrobacterium*-mediated transformation and microprojectile transformation systems in watermelon for establishing the best conditions for transformation system in watermelon.

٣- To introduce the plant defensin gene into the Egyptian watermelon cultivars for acquiring resistance for fungal infection.

٤- To confirm the integration and expression of the transgenes in the putative transgenic plants by molecular analysis.