

TRANSFER OF GLUCANASE GENE TO RESIST LATE BLIGHT DISEASE IN POTATO

By

HEBA SAYED SHEBL

B.Sc. Agric. Sci. (Genetics), Ain Shams University, 2008

A Thesis Submitted in Partial Fulfillment

of

The Requirements for the Degree of

Master of sciences

In

AGRICULTURAL SCIENCES

(Genetics)

Department of Genetics

Faculty of Agriculture

Ain Shams University

2017

Approval Sheet

**TRANSFER OF GLUCANASE GENE TO RESIST
LATE BLIGHT DISEASE IN POTATO**

By

HEBA SAYED SHEBL

B.Sc. Agric. Sc. (Genetics), Ain Shams University, 2008

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

Dr. Abdel-Raheem Tawfeek Abdel-Reheem

Prof. Emeritus of Genetics, Faculty of Agriculture, El Mania University

Dr. Abdel-Fatah Abdel-Kader Mohamed Awad

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

Dr. Mahmoud Ahmed Abd El-Hafiez Sallam

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

Dr. Mohamed Abdel-Salam Rashed

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

Date of Examination: 25 / 3 / 2017

TRANSFER OF GLUCANASE GENE TO RESIST LATE BLIGHT DISEASE IN POTATO

By

HEBA SAYED SHEBL

B.Sc. Agric. Sci. (Genetics), Ain Shams University, 2008

Under the supervision of:

Dr. Mohamed Abdel-Salam Rashed

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

Dr. Mahmoud Ahmed Abd El-Hafiez Sallam

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

Dr. Emad Anis Metry

Head Research of Genetics, Agriculture Genetics Engineering Research
Institute Agriculture Research Center

ABSTRACT

Heba Sayed Shebl Mohammed. Transfer of Glucanase Gene to Resist Late Blight Disease in Potato. Unpublished M.Sc., Thesis, Department of Genetic, Faculty of Agriculture, Ain Shams University, 2017.

Potato late blight is a pandemic disease caused by the highly virulent *Phytophthora infestans* fungus. Two different types of explants (internode and leaf) were cultured for induction and regeneration from three potato cultivars (Spounta, Diamont and Desiree). The highest value of Desiree leaf explants was 80% induced from develop callus in 2.4.Dmedia. The best value of maximum shoot regeneration was 90.3 for Spounta in NAA and 4mg/L KIN media from internode and 88.4 for Desiree in BA, IAA and GA3 media from leaf proved to be more effective. Cloning of glucanase gene in pRI 201-AN plasmid which carrying kanamycin resistance (nptII) gene were performed and followed by transformation in *Agrobacterium tumefaciens* strain LBA4404 which used for plant transfection. Nucleotide and amino acid sequences of transformed *Agrobacterium* were analyzed. The putatively transgenic plants were confirmed using polymerase chain reaction (PCR) for genomic and cDNA.

Keywords: Potato, Late blight, Glucanase gene

ACKNOWLEDGEMENT

First of all, I would like to express my deepest thanks to “ALLAH” for helping me to carry out and complete this work.

I wish to express my deep gratitude and sincere appreciation to **Prof. Dr. Mohamed Abdel El Salam Rashed.**, Professor of Genetics, Genetics Dept., Faculty of Agriculture, Ain Shams University for his continuous supervision, tremendous help, kind encouragement, sincere help criticism, facilities offered and valuable help while writing and revising this thesis. Great appreciation is also expressed to **Prof. Dr. Mahmoud Ahmed Abd El-Hafiez** Prof. of Genetics, Faculty of Agriculture, Ain Shams University for his continuous supervision, precious advices and for revising this thesis. I wish to express my deep gratitude and sincere appreciation to **Prof. Dr. Emad Anis Metry**, Deputy Director for Research, Agriculture of Genetic Engineering Research center, for continuous supervision, kind encouragement, precious advices during the progress of thesis work and the preparation and writing of the manuscript.

I would also like to express my deepest thanks and everlasting gratitude to **Prof. Dr. Ahmed mohammed**, Professor of Genetics, Agriculture Genetics Engineering Research Institute (AGERI).

I would also like to express my deepest thanks and everlasting gratitude to, **Mrs Mervet** for helping me in my work.

Many thanks to my colleagues; my lab mates, staff of Micro Propagation Technology Lab and friends whose timely help needs special mention., Agriculture Genetic Engineering Research Institute (AGERI), for their great help, encouragement and real cooperation during the progress.

Finally, I am indebted to my family for their great help and patience during this work, especially the mother, sister, brother and my husband for continuous encouragement and praying for me.

CONTENTS

	Page
LIST OF TABLES	VIII
LIST OF FIGURES	Vii
LIST OF ABBREVIATIONS	ix
I. INTRODUCTION	1
II. REVIEW OF LITERATURE	4
1. Regeneration in potato	4
2. Biotic and a biotic stress combinations	5
2.1. Late blight history	6
2.2. <i>Phytophthora infestans</i> is causal organism	6
2.3. Symptoms of late blight	7
3. Defence mechanisms	8
4. Glucanase	10
4.1. Possible Role of β -1, 3-Glucanases in Plant Defense	11
4.2. Classification of Plant β -1, 3-Glucanases	12
4.4. β -1, 3-Glucanase Genes in Transgenic Plants	13
5. <i>In vitro</i> transformation	13
5.1 The transformation of potato plants	14
5.2. The Mechanism of DNA Transfer	15
6. Molecular analysis of putative transgenic plants	15
6.1. Polymerase chain reaction (PCR)	15
6.2. Reverse transcription-polymerase chain reaction,	16
III. MATERIALS AND METHODS	18

II

I. Materials	
1.1 Plant Materials	18
1.2 Bacteria	18
2. Bacteriological media	18
2.1 Luria- Bertani broth (LB)	18
2.2 LB Agar medium	18
2.3 S.O.C. Medium	18
3. Supplements	19
3.1. Ampicillin	19
3.2 kanamycin	19
3.3 cefotaxine	19
3.4 X-gal	19
3.5 IPTG	19
3.6 Restriction Enzymes	19
4. Vectors	20
4.1 Plasmid pRI 201-AN was used in this study	20
4.2 A pGEM-T easy vector was obtained from (Promega)	20
5. DNA solutions	21
5.1 50X TAE buffer	21
5.2 Ethidium bromide	22
6. Computer software	22
7. DNA primers	22
2- Methods	
2.1 Regeneration from callus	23
2.2 Gene cloning	23
2.2.1 Miniprep plasmid DNA system for glucanase gene and expiration vector	23
2.2.2 Polymerase chain reaction (PCR) for Glucanase gene	25
2.3 Cloning of PCR Products into pGEM®-T Easy Vectors	25
2.2.4 Transformation of β -1, 3 glucanase (GLN) gene	25

III

intoDH10B high efficiency competent Cells	
2.2.5 Confirm of cloned glucanase gene into A pGEM-T easy vector	26
2.2.6 EcoRI restriction enzyme digestion	26
2.2.7 SalI restriction enzyme digestion	27
2.2.8 Plasmid purification	28
2.2.9 NdeI restriction enzyme digestion	29
2.2.10 Plasmid purification	29
2.2.11 Ligation of DNA	29
2.2.12 Transformation of pRI 201-AN plasmid into DH10B cell	30
2.2.13 Preparation of <i>Agrobacterium</i>	30
2.2.14 Transformation of plasmid into <i>Agrobacterium tumefaciens</i> competent cells	31
2.2.15 Sequencing and analysis of Glucanase gene.	31
2.3. Transformation of desiree potato cultivar via <i>Agrobacterium</i>	31
2.3 .1 Regeneration from callus	32
2.3.2 DNA extraction from transgenic desiree potato cultivars	32
2.3.3 Polymerase chain reaction (PCR) analysis.	33
2.3.4. Reverse transcription polymerase chain reaction	33
a. RNA isolation from transgenic Desiree potato plants	24
IV.RESULTS AND DISCUSSION	36
1 PCR amplification of glucanase gene	36
2 Inserted of glucanase gene into pGEM-T easy vector.	37
3 Inserted of glucanase gene into PRI 201-the plant expression vector.	39
4 Transformation of <i>Agrobacterium tumefaciens</i>	41

5. Nucleotide and amino acid sequence analysis of Glucanase gene.	42
6. Optimization of regeneration system in potato.	45
6.1. Plant regeneration capacity among potato cultivars	45
7. Shoots proliferation and development on regeneration medium of potato plants cultivar Desiree.	48
7.1. Molecular analysis of putative transgenic plants by using PCR and RT-PCR analysis.	50
7.1.1 PCR detection for transformed potato plants	50
7.1.2 RT-PCR detection for transformed potato plants	51
SUMMARY	54
IIV REFERENCES	56
	71

الملخص العربي

LIST OF TABLES

Table No.	Page
Primer sequence, number of base, expected product for glucanase gene (GLN), pRI 201-AN vector (pRI) and NPT II gene (Kan).	24
Regeneration of potato (<i>Solanum tuberosum</i> L.) cultivars Desiree, Spunta and Diamont	57
Regeneration and transformation percentages of potato (<i>Solanum tuberosum</i> L.) cultivars of Desiree control and transgenic plant	60

LIST OF FIGURES

Fig. No.		Page
1	Restriction map and general specification of Plasmid pRI201-AN	20
2	Restriction map and general specification of pGEM-T easy vector.(B)sequence of restriction enzyme site of pGEM-T easy vector , the arrow indicates the start of the transcription of the T7 RNA polymerase	21
3	A flowchart showing cloning strategy of PCR amplified Gln- coding Sequence (1020 bp) in pGMT easy vector	27
4	A flowchart showing Cloning strategy of Digested Glucanase gene in pGEM®-T Easy Vector by Restriction enzyme and insert it in pRI 201-AN plant expression vector	29
5	Amplified 1020bp fragment size of glucanase gene	37
6	Restriction digestion of recombinant clone using restriction enzyme EcoR1 M = 1 Kb ladder. Lane 1 =1020bp fragment size (Glucanase gene).Lane 2 = vector non digested with Glucanase gene	38
7	Integration of glucanase gene into pGEM-T easy vector, as confirmed by the presence of the 1020 bp PCR-amplified glucanase fragment. M =1 Kb ladder: Lane 1 to 4= fragment of glucanase gene	39
8	Integration of glucanase gene into pRI 201-ANvector, as confirmed by the presence of the 1020 bp PCR-amplified glucanase fragment. M =1 Kb ladder: Lane 1 to 10= fragment of glucanase gene: Lane11: negative control	40
9	Integration of glucanase gene into pRI 201-	41

Fig. No.		Page
	ANvector, as confirmed by using pRI 201-AN (reverses) and glucanase gene (forward) primer. M =1 Kb ladder: Lane 1 to 10= fragment of glucanase: Lane 11: negative control	
10	. Agarose gel for PCR product of 1020bp fragment size glucanase gene from Transformed <i>agrobacterium</i> M: 1 Kb ladder; Lane 1: negative control; Lane 2: positives (glucanase gene in pRI 201-ANvector) ; Lane 3 to 4: fragment of glucanase	42
11	The nucleotide sequence and the predicted amino acid sequence of glucanase gene	43
12	Sequences producing significant alignments	44
13	Callus formation from leaf (a,b), Internode (c,d) explants: Multiple shoot formation with Medium (I) and Medium (II) of potato plants cultivar Diamant, Spunta and Desiree, ; hardening under glass house condition	46
14	Colum's values show the differences in shoot percentage among three potato cultivars	47
15	Callus induction and plant regeneration from leaf explants of Desiree potato cultivars	49
16	A garose gel for PCR product: 1020bp fragment size of glucanase gene from the transgenic potato lines using glucanase primers. M: 1 Kb ladder. Lane 1: non transgenic potato line. Lane 2: (clone2). Lane 3 to 6: transgenic potato lines	50
17	Agarose gel for PCR product: 650bp fragment size of glucanase gene from the transgenic potato lines using NPT II primers. Lane M : 1Kp DNA ladder, Lane 1 to 2: Transgenic potato lines, Lane 3: Non transgenic potato lines	51

Fig. No.		Page
18	Verification of glucanase gene presence and incorporation into putatively transgenic potato plants via RT-PCR analysis. Screening plants showed the expected 1020bp band in several individuals. Lane M: 100bp DNA ladder (gene ruler™Fermentas), Lane1: negative control, Lane (2): represented putatively transgenic plants (positive). Lane (3, 4): transformed potato plants	52
19	Verification of glucanase gene presence and incorporation into putatively transgenic potato plants via RT-PCR analysis. Screening plants showed the expected 450bp band in several individuals. Lane M: 100bp DNA ladder (gene ruler™Fermentas), Lane1: negative control, Lane (2): represented putatively transgenic plants (positive). Lane (3, 4): transformed potato plants	53

LIST OF ABBREVIATIONS

BA	: Benzyl amino purine
Bp	: Base pair
° C	: Celsius degree
CaCl ₂	: Calcium chloride
c DNA	: Complementary synthesized from a mRNA
cm	: Centimeter
dd H ₂ O	Distilled deionized water
DNA	: Deoxyribonucleic acid
DNA ase	deoxyribo nuclease enzyme that catalyzesThe hydrolyticcleavage of phosphodiesterlinkages in the DNA backbone.
2,4-D	: 2,4-dichlorophenoxyacetic acid
<i>et al.</i>	Et alia
IAA	Indole acetic acid
<i>in vitro</i>	: In an artificial environment outside the livingorganism
Kb	: kilobase
Kinetin	: Furfuryl amino purine
LB Luria	: Bertani media
M	: Molar
Min	: Minute
Mg	: Milligram
µg	: Microgram
ml	: Milliliter
µL	: Microliter
mM	: Millimolar
mm	: Milimeter
mRNA	: Messenger ribonucleic acid
MS	Murashige and Skoog (1962)
NaOH	: Sodium hydroxide
<i>NptII</i>	: Neomycin phosphor transferase gene

O. D.	: Optical density
PCR	: Polymerase Chain Reaction
P35S	: Cauliflower mosaic virus 35S promoter
pH	: Per hydrogen
RNA	: Ribonucleic acid
Rpm	: Revolutions per minute
RT-PCR	: Reverse transcription polymerase chain reaction
TAE	: buffer solution containing Tris base, acetic acid and EDTA
Ti plasmid	: Tumor inducing plasmid
UV	: Ultraviolet
%	: Percentage