DEVELOPMENT OF GENETIC TRANSFORMATION SYSTEM IN OKRA (ABELMOSCHUS ESCULENTUS (L). MOENCH)

Submitted by
Dur-E -Aeman

15001254013

Supervised by
Dr. Nouman Rasool

Department of life sciences
School of science
University of management and technology
Lahore, Pakistan

2017

DEVELOPMENT OF GENETIC TRANSFORMATION IN OKRA

(ABELMOSCHUS ESCULENTUS (L). MOENCH)

Submitted to University of Management and Technology Lahore

In partial fulfillment of the requirements

For the award of degree of

MS

BIOTECHNOLOGY

By

Dur-E-Aeman

ID

1	5	0	0	1	2	5	4	0	1	3

Session: 2015- 2017

DEPARTMENT OF LIFE SCIENCES

SCHOOL OF SCIENCE

UNIVERSITY OF MANAGEMENT AND TECHNOLOGY,

LAHORE, PAKISTAN

DECLARATION

I, Dur-E-Aeman student of MS biotechnology ID: 15001254013 aware of and understand the university's policy on plagiarism and I certify that this thesis titled **"Development of genetic transformation system in okra *(Abelmoschus esculentus(L). M*oench)"** is my own work, except where indicated by referencing, and the work presented in it has not been submitted in support of another degree or qualification from this or any other university or institute of learning. All experimental work belong to me; the collaborative contributions have been indicated clearly and acknowledged. Due references have been provided on all supporting literature and resources.

Dated: _____ _____

Dur-E-Aeman

Similarity Report(Signed by CLO/UMT Officials)

RESEARCH COMPLETION CERTIFICATE

This is to certify that the thesis entitled **"Development of genetic transformation system in okra *(Abelmoschus esculentus (L). Moench*)"**submitted by **Dur-E-Aeman** ID: **15001254013** has been accepted towards the partial fulfillment of the requirement for MS degree in Biotechnology.The quantum and quality of the work contained in this thesis is adequate for the award of thedegree.

_____ _____

Supervisor **External Examiner**

_____ _____

Dr. Nouman Rasool
Chairman,
Department of Life Sciences,
UMT, Lahore

Dr. Muhammad Azhar Iqbal
Dean
School of Science,
UMT, Lahore

ABSTRACT

This study appraises the development of genetic transformation system in okra (*Abelmoschusesculentus L. Moench).* Okra plays an important role in human diet and is an excellent source of carbohydrates, proteins, fats and minerals. Okra is affiliated with Malvaceae ancestors and is highly susceptible towards insects and viruses. Reported begomoviruses of okra reported throughout the world are *Bhendi yellow vein mosaic virus, Okra yellow vein mosaic virus, Okra yellow crinkle virus,Okra yellow mosaic Mexico virus and Cotton leaf curl Alabad virus.* Conditions were optimized by establishing a simple regeneration system of okra. 193 embryos were isolated and 69% total regeneration frequency was calculated. Best regeneration frequency was calculated when embryos were grown on MS medium containing 1g GA/L and B5 vitamin. 342 embryos were isolated for development of transgenic plants by using embryo as explant. The transformation efficiency was 2.92 %. Presence of transgene was confirmed by Green fluorescent protein (GFP) expression and PCR analysis. Expression of GFP were effortlessly and rapidly distinguished when examined under a fluorescence microscope at 40X- 100X resolving power. Microscopic analysis was done by using fluorescence stereo microscope to check GFP expression. A tissue sample of okra plant leaf was placed on glass slide. Expression of Green fluorescent protein genes was quickly distinguished at 40-100X resolution power. PCR analysis confirmed the presence of RNAi constructs in transgenic plants of okra when amplified PCR product of size 417 bp was visualized on 1 % agarose gel stained with ethidium bromide.

ACKNOWLEDGEMENT

Allpraises and thanks are for Almighty Allah, The Rehman, the Rahim, the only Creator of the whole universe, the Supreme, Self-existing, who blessed me sound health, thoughts, talented teachers, cooperative friends to carry out this research work. Then I pay all my respect to the greatest man of the universe, the Holy Prophet Muhammad (SAW) for whom Allah created the whole universe.

I wish to express my deep sense of gratitude to my supervisor **Dr. Nouman Rasool (**Chairperson Department of Life Sciences, University of Management and Technology). His skillfuladvice, sincere cooperation and guidance enabled me to complete this research work successfully. It is my privilege and honor to express my sincere attributes to **Dr.Muhammad Shafiq,** Assistant Professor, Institute of Agricultural Sciences, University of the Punjab, Lahore.Special thanks to him for his patience motivation, enthusiasm, and providing me with immense knowledge.

I am highly obliged to my lab colleagues at IAGS: **MissBarrera Maryam, Miss Mubashra, Miss Farah Saeed and Mr Sohaib**. Their accommodative and friendly behavior made my work less laborious. I would like to express my deepest gratitude to my respected parents, my brother **Mr Dawood**, my sisters, **MissKashuf** and**Miss Zainab** for giving me the power to overcome all the challenges and difficulties in my work. I have no words that can suffice the role of my friends **Miss Ayesha Sadiqa**, **MissRabbia Muzaffar** and **Miss Samra Naseer**who made my journey beautiful. I would never forget all the beautiful moments I shared with them.

TABLE OF CONTENTS

TABLE OF CONTENTS i

LIST OF TABLES v

LIST OF FIGURES vi

ABBREVIATIONS viii

CHAPTER 1

INTRODUCTION.....1

1.1Viruses: 2

1.2 Plant Viruses: 4

1.3 Gemini Viruses: 4

1.4 Classification of Gemini viruses: 6

1.4.1Begomoviruses: 6

1.4.1.1Bipartite begomoviruses: 6

1.4.1.2Monopartite Begomoviruses: 7

1.4.2 *Mastrevirus*: 8

1.4.*3 Curtovirus:* 9

1.4.4 *Topocuvirus:* 9

1.4.5 *Eragrovirus:* 10

1.4.6 *Turncurtovirus:* 10

1.4.7 *Becurtovirus:* 11

1.4.8*Capulavirus*…… 11

1.4.9*Grabulovirus*…… *12*

1.4.8 Reported Begomoviruses of Okra: .....

1.5Genetic Transformation: 14

1.5.1Direct DNA Transformation:.....12

1.5.2 Electroporation:.....15

1.5.3 PEG Mediated Transformation:.....16

1.5.4 Mechanical Transformation:.....16

1.5.4.1Microinjection:.....16

1.5.4.2Particle Bombardment:.....17

1.6 *Agrobacterium* Mediated DNA Transformation:.....17

1.7Green Fluorescent Protein:.....18

Chapter 2

REVIEW OF LITERATURE.....21

Chapter 3

MATERIALS AND METHODS.....35

3.1 Selection of Plant:.....35

3.2 Procurement of Gene Construct:.....35

3.3 Culture Medium Preparations:.....35

3.3.1LB Medium (Lauria Bertani) for 1 Litre:.....35

3.3.2 Pouring of Agar Plate:.....35

3.4 Preparation of Competent Cells:.....36

3.4.1Preparation of Heat Shocked Competent *Escherichia coli* (DH5α) Cells:.....36

3.4.2Preparation of Competent *Agrobacterium Tumefaciens* Cells:.....37

3.5 Transformation of Competent cells:.....37

3.6 Transformation of Competent *Agrobacterium Tumefaciens* Cells:.....37

3.7 Preparation of Glycerol Stocks:.....38

3.8 Establishment of Regeneration System of Okra: 38

3.8.1Seed Sterilization: 38

3.8.2Establishment of Genetic Transformation system in Okra: 39

3.9 Regeneration of Transgenic Okra Plants: 39

3.9.1Sterilization of Seeds: 39

3.9.2 Isolation of Embryos: 40

3.9.3 Inoculum Preparation: 40

3.9.4 Co-Inoculum and Shoot and Root Development of Putative Transgenic Okra Plants: 40

3.9.5 Sub Culturing for Regeneration of Transgenic Plants of Okra: 41

3.9.6 Acclimatization: 41

3.10 Confirmation of GFP and RNAi Construct in Transgenic Plants of Okra: 42

3.10.1Analysis of GFP Expression: 42

3.10.2 Confirmation of RNAi Construct through PCR: 42

3.10.3DNA Isolation by CTAB Method: 42

3.10.4PCR Analysis: 43

3.10.5 Agarose-Gel Electrophoresis: 44

Chapter 4

RESULTS 45

4.1 Germination of Seeds: 45

4.2 Regeneration of Shoots and Roots: 45

4.3 Regeneration of Okra Plants: 47

4.4 Transformation Steps: 49

4.4 Confirmation of GFP and RNAi Construct in Transgenic Plants of Okra: 51

4.4.1 Analysis of GFP Expression in Transgenic Plants of Okra

(*Abelmoschus esculents*): 51

4.4.2PCR Analysis: 52

Chapter 5

DISCUSSION 53

Chapter 6

REFERENCES.....60

LIST OF TABLES

Table No.	Title	Page No.
Table 3.1:	Optimization of Conditions for Regeneration of Okra Plants:	39
Table 3.2:	Preparation of Master Mix for PCR	43
Table 4.1:	Regeneration Frequency of Roots of Non-Transgenic Plants of Okra	45
Table 4.2:	Total Regeneration Frequency of Non-Transgenic Plants	47
Table 4.3:	Transformation Efficiency of Transgenic Plants	50
Table 4.4:	Transformation Efficiency	50

LIST OF FIGURES

Figure. No	Titles	Page No.
Figure 1.1	A) Healthy okra plant (B) Diseased okra plant	2
Figure 1.2	Schematic structure of virus (Picture taken from autocww.24ContentFiles/ Virus.html	3
Figure 1.3	The icosahedral configuration of a plant *geminivirus* (Hipp *et al.*, 2017).	5
Figure 1.4:	The genome organization of bipartite *begomovirus*: the positions ofORFs of genomic components are shown by arrows. The open reading frames in DNA-A code for replication Rep protein, TrAPREn, CP, AV2 and AC4 protein involved in pathogenicity. Open reading frames in DNA-B code for MP and NSP. Open blocks represent the intergenic region (Padidam *et al.*, 1995).	7
Figure 1.5:	Genome organization of isolates in various Geminiviruses lineages (LIR, long intergenic region; SIR, short intergenic region; CR, common region; rep, replication-associated protein; ren, replication enhancer; trap, transactivator protein; ss, silencing suppressor; tags, transcriptional gene silencing; sd, symptom determinant; cp, capsid protein; mp, movement protein; reg, regulatory gene; NSP, nuclear shuttle protein. Monopartitegeminiviruses and DNA-A components of bipartite begomoviruses contain (V1, V2, V3 and V4 regions that encode CP and are involved in cell to cell movement, C1 is involved in viral replication, C2 and C3 involved in post-transcriptional modifications. DNA-B components of bipartite begomovirusesconsist of (AVI) involved in the movement, AC1, AC2, AC3, AC4 are involved in gene silencing, BV1 involved in movement and BC1 is responsible for viral replication (Costa, 1976).	13

Figure. No	Titles	Page No.
Figure 4.1:	(A) Germinated seeds (B) Isolated embryos	45
Figure 4.2:	Formation of multiple shoot buds on MS selection medium after 10 days of incubation.	46
Figure 4.3:	Development of roots in rooting media.	47
Figure 4.4:	Development of plants after 3 months of selection. After 3-month different stages of plant	48
Figure 4.5:	Transformation steps (A) Incubated seeds (B) Isolation of embryos (C) Isolated embryos (D) Injury to plulmle of embryos (E) Incubation of embryos on MS plates (F) Plants in shooting media (G) Plants in rooting media (H) After 3 months different stages of plants(I) Acclimatization	49
Figure 4.6:	Fluorescence analysis of GFP expression in a transgenic plant of Okra (Abelmoschus esculentus (L) Moench). (A): Control plant tissue without GFP (B)Transgenic plant tissue expressing GFP, (C) Transgenic plant tissue at 100X (magnification), (D)Transgenic plant tissue at 40X, (E) Transgenic plant tissue at 10X, (F)In a single cell at 100X.	51
Figure 4.7:	Lane1, 2, 3 and 4 confirmed the presence of RNAi constructs in transgenic plants of okra.	52

ABBREVIATIONS

µL	microlitre
$CaCl_2$	calcium chloride
CLCuD	cotton leaf curl disease
CP	coat protein
CR	common region
CTAB	cetyl trimethyl ammonium bromide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
dsDNA	double-stranded DNA
dsRNA	double-stranded RNA
HR	hypersensitive response
ICTV	international committee on taxonomy of viruses
IPTG	isopropyl-beta-D-1-thiogalactopyranoside
IR	intergenic region
LB	Lauria Bertani
LIR	large intergenic region
mg	milligram
$MgCl_2$	magnesium chloride
mM	millimolar
MP	movement protein
NaCl	sodium chloride
NaOH	sodium hydroxide
ng	nanogram
NLS	nuclear localization signals
NSP	nuclear shuttle protein
nt	nucleotide
NW	New World

OD	optical density
ORF	open reading frame
OW	Old World
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
pH	paviour of hydrogen
PTGS	post-transcriptional gene silencing
RBR	retinoblastoma-related protein
RCA	rolling circle amplification
REn	replication enhancer protein
Rep	replication-associated protein
RNA	ribonucleic acid
rpm	revolutions per minute
SCR	satellite conserved region
SDW	sterile distilled water
SIR	small intergenic region
ssDNA	single-stranded DNA
ssRNA	single-stranded RNA
TAE	tris-acetate EDTA
Taq	*Thermus aquaticus*
TGS	transcriptional gene silencing
TrAP	transcriptional activator protein
UV	ultraviolet
X-Gal	5-Bromo-4-chloro-3-indolyl-b-D-galactopyranoside
GA	Gibberelic Acid
IAA	Indole-Acetic-Acid
MS	Murashige and Skoog
LB	Luria Bertani
GFP	Green Fluorescent protein

Viruses and Satellites

African cassava mosaic virus	(ACMV)
Beet curly top virus	(BCTIV)
Beet curly top Iran virus	(BCTIV)
Bean golden mosaic virus	(BGMV)
Bean golden yellow mosaic virus	(BGYMV)
Bhendi yellow vein mosaic virus	(BYVMV)
Chilli leaf curl beta satellite	(ChLCB)
Chilli leaf curl virus	(ChLCV)
Cotton leaf curl beta satellite	(CLCuB)
Cotton leaf curl Multan virus	(CLCuMuV)
Cotton leaf curl Rajasthan virus	(CLCuRaV)
Horseradish curly top virus	(HCT)
Maize streak virus	(MSV)
Spinach curly top Arizona virus	(SCTAV)
Spinach severe curly top virus	(SSCTV)
Tobacco mosaic virus	(TMV)
Tobacco yellow dwarf virus	(TbYDV)
Tomato golden mosaic virus	(TGMV)
Tomato leaf curl New Delhi betasatellite	(ToLCNDB)
Tomato leaf curl New Delhi virus	(ToLCNDV)

Tomato leaf curl Pakistan alphasatellite	(ToLCPKA)
Tomato leaf curl virus	(ToLCV)
Tomato mottle virus	(ToMoV)
Tomato pseudo-curly top virus	(TPCTV)
Tomato yellow leaf curl Oman betasatellite	(TYLCuOB)
Tomato yellow leaf curl virus	(TYLCV)
Turnip curly top virus	(TCTV)
Wheat dwarf virus	(WDV)

Chapter 1

INTRODUCTION

Abelmoschusesculentus L. Moench is commonly known as okra, a significant legume within the different terrain. Okra is farmed in humid, subtropical and tepid latitudes zone around the globe (Koh *et al.,* 2008). Okra is identified by avariety of confined names throughout the globe. It is the member of Malvaceae ancestors. Outside of the US and Great Britain, it is known as lady's finger and in Pakistan and India, it is known by a variety of names i.e bhindi and vindi.