

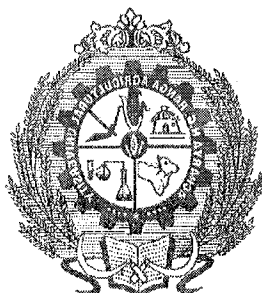
**STUDIES ON DEVELOPMENT OF TRANSGENIC MALE
STERILE AND RESTORER LINES IN SAFFLOWER
(*Carthamus tinctorius* L.) USING UNEDITED
MITOCHONDRIAL GENE(S)**

By

K.N.YAMINI

M. Sc. (Ag)

THESIS SUBMITTED TO THE
ACHARYA N. G. RANGA AGRICULTURAL UNIVERSITY
IN PARTIAL FULFILMENT OF THE REQUIREMENTS
FOR THE AWARD OF THE DEGREE OF
DOCTOR OF PHILOSOPHY
IN THE FACULTY OF AGRICULTURE



ANGRAU Central Library
HYDERABAD-500 030.
ACC. No. D-8088.
Date 9-1-08

DEPARTMENT OF GENETICS AND PLANT BREEDING
COLLEGE OF AGRICULTURE
ACHARYA N.G.RANGA AGRICULTURAL UNIVERSITY
RAJENDRANAGAR, HYDERABAD- 500 030
OCTOBER, 2007

CERTIFICATE

Mrs. **K. N. Yamini** has satisfactorily prosecuted the course of research and that the thesis entitled **“STUDIES ON DEVELOPMENT OF TRANSGENIC MALE STERILE AND RESTORER LINES IN SAFFLOWER (*Carthamus tinctorius* L.) USING UNEDITED MITOCHONDRIAL GENE(S)”** submitted is the result of original research work done and is sufficiently high standard to warrant its presentation to the examination. I also certify that the thesis or part there of has not been previously submitted by her for a degree of any University.

Date : 24.10.2007
Place: Hyderabad


(Dr. S. Sokka Reddy)
Chairman of Advisory Committee

CERTIFICATE

This is to certify that the thesis entitled “STUDIES ON DEVELOPMENT OF TRANSGENIC MALE STERILE AND RESTORER LINES IN SAFFLOWER (*Carthamus tinctorius* L.) USING UNEDITED MITOCHONDRIAL GENE(S)” submitted in partial fulfillment of the requirements for the degree of **Doctor of Philosophy in Agriculture** of the **Acharya N. G. Ranga Agricultural University, Hyderabad**, is a record of the bonafide research work carried out by **Mrs. K. N. Yamini** under our guidance and supervision. The subject of the thesis has been approved by the Student’s Advisory Committee.

No part of the thesis has been submitted for any other degree or diploma. The published part has been fully acknowledged. All assistance and help received during the course of the investigation have been duly acknowledged by the author of the thesis.

S. Sokka Reddy

(Dr. S. Sokka Reddy)

Chairman of the Advisory Committee

Thesis approved by the Student’s Advisory Committee

Chairman: **Dr. S. Sokka Reddy**
Professor (Biotechnology)
College of Agriculture,
ANGRAU, Rajendranagar
Hyderabad-500030

S. Sokka Reddy
24.10.2007

Co-Chairman: **Dr. V. Dinesh Kumar**
Senior Scientist (Biotechnology)
Directorate of Oilseeds Research
Rajendranagar, Hyderabad-500030

V. Dinesh Kumar

Member: **Dr. M. Sujatha**
Senior Scientist (Genetics and Cytogenetics)
Directorate of Oilseeds Research,
Rajendranagar, Hyderabad-500030

M. Sujatha

Member: **Dr. T. Y. Madhulety**
Professor (Plant Physiology)
College of Agriculture,
ANGRAU, Rajendranagar
Hyderabad-500030

T. Y. Madhulety

Member: **Dr. P. V. Rao**
Professor (Plant Physiology)
College of Agriculture,
ANGRAU, Rajendranagar
Hyderabad-500030

P. Venkateshwar Rao

CONTENTS

Chapter No.	Title	Page No.
I	INTRODUCTION	1-4
II	REVIEW OF LITERATURE	5
2.1	Male sterility systems in safflower	6
2.2	Safflower hybrids in India	10
2.3	Molecular basis of CMS	11
2.4	Transgenic male sterility	12
2.5	Requirements for induction of transgenic male sterility	18
2.6	Different strategies for induction of transgenic male sterility	27
2.7	Restoration of fertility in transgenic male sterile lines	42
2.8	Tissue culture of safflower	48
2.9	Genetic transformation of safflower	50
2.10	Cloning and transformation initiatives in safflower	51
2.11	Analysis of transgenic plants	52
III	MATERIALS AND METHODS	58
3.1	Materials	59
3.1.1	Plant Material	59
3.1.2	Equipment	59
3.1.3	Media	61
3.1.4	Hormones	61
3.1.5	Antibiotics/Herbicide	61
3.1.6	Other chemicals and consumables	64
3.1.7	Vectors and Strains	64
3.2	Methods	64
3.2.1	Development of gene constructs	64
3.2.2	Tissue culture and transformation	103

Chapter No.	Title	Page No.
IV	RESULTS	115
4.1	Identification of suitable mitochondrial genes by RNA editing studies	115
4.2	Standardizing the shoot regeneration in safflower through embryogenic pathway	144
4.3	Cloning of the identified mitochondrial gene(s) in appropriate orientation to develop the sense and antisense constructs	156
4.4	<i>Agrobacterium</i> -mediated transformation of safflower and tobacco with the developed constructs	181
4.5	Confirmation of the developed safflower and tobacco transgenics	206
V	DISCUSSION	231
5.1	Male sterility systems in safflower	231
5.2	Transgenic male sterility	232
5.3	Identification of mitochondrial genes that undergo substantial RNA editing in safflower	235
5.3.1	RNA editing in <i>atp6</i>	235
5.3.2	Editing in <i>nad3</i> and <i>atp9</i>	236
5.4	Development of constructs for induction of male sterility	242
5.5	Development of constructs for restoration of fertility	246
5.6	Mobilization of the recombinant binary vectors into <i>Agrobacterium</i>	249
5.7	Tissue culture in safflower	250
5.8	Transformation of safflower	253
5.9	Transformation of tobacco	255
5.10	Analysis of the transgenic plants	256
VI	SUMMARY	263-267
	LITERATURE CITED	268
	APPENDICES	285-294

LIST OF TABLES

Table No.	Title	Page No.
2.1	Examples of mitochondrial sequences/genes associated with CMS	13
2.2	Examples of induction of transgenic male sterility using different approaches	21-25
3.1	Equipments used in the present study	60
3.2	Composition of Murashige and Skoog medium	62
3.3	Details of hormones used for tissue culture	63
3.4	Details of the antibiotic/herbicide used for tissue culture	63
3.5	Salient features of the commercial vectors used	70
3.6	Other recombinant vectors utilized for cloning purpose	70
3.7	Details of the sequences isolated and/or utilized	75
3.8	Details of the primers used in the present study	77-78
3.9	Details of the clones developed in the present study	83-85
3.10	Different primary media combinations tried for immature embryo culture	105
3.11	Different secondary media combinations tried for immature embryo culture	105
3.12	Different media combinations used during tissue culture	106
3.13	Media along with the antibiotics/herbicides used for selection of transformants at different stages	107
3.14	Primers used and amplicons obtained during confirmation of transgenics by PCR analysis	112
4.1	Amplicons obtained with different primer combinations for <i>atp6</i>	119
4.2	Summary of RNA editing and encoded amino acid changes for <i>nad3</i> gene of safflower	130
4.3	Summary of RNA editing and encoded amino acid changes for <i>atp9</i> gene of safflower	134
4.4	Response of immature embryos on primary media containing different concentrations of TDZ with or without NAA	145

Table No.	Title	Page No.
4.5	Response of immature embryos on primary media containing different concentrations of 2,4-D	148
4.6	Response of immature embryos on five secondary media combinations	151
4.7	Response of different sized immature embryos on four media combinations: Explants forming adventitious shoots	194
4.8	Transformation frequency in safflower for the different constructs	198
4.9	Frequency of root induction in the safflower transgenics	201
4.10	Details of the safflower transgenics	201
4.11	Transformation frequency in tobacco for the different constructs	207
4.12	Details of the tobacco transgenics	207
4.13	PCR analysis of transgenic shoots at the tissue culture level	208
4.14	Analysis of flowered safflower TCNN-Bar and TCAN-Bar transgenics	209
4.15	Analysis of flowered tobacco TCNN-Bar transgenics	208
4.16	Analysis of flowered tobacco TCAN-Bar transgenics	209
4.17	Analysis of flowered tobacco T-ihp <i>nad3</i> transgenics	209
4.18	Analysis of flowered tobacco T-ihp <i>atp9</i> transgenics	210

LIST OF ILLUSTRATIONS

Figure No.	Title	Page No.
3.1a	Schematic diagram of T/A cloning vector	65
3.1b	Multiple cloning sites (MCS) of T/A vector	65
3.2	Schematic diagram of pUC18 vector	66
3.3	Schematic diagram of pRT100 vector	67
3.4	Plasmid map of pCAMBIA0390 vector	68
3.5	Plasmid map of pCAMBIA1300 vector	69
3.6	Map of the pBARGUS vector	71
3.7	Plasmid map of pCAMBIA1305.2-TCON vector	72
3.8	Map of generic vector pRT100-Cat Int	73
3.9	Schematic representation of development of TCNN-Bar and TCAN-Bar constructs in pCAMBIA0390	92
3.10	Plasmid map of the developed pBin-TCNN-Bar	94
3.11	Schematic representation of development of full-length antisense <i>nad3</i> and <i>atp9</i> constructs in pRT100	95
3.12	Schematic representation of development of ihp <i>nad3</i> and ihp <i>atp9</i> constructs in pRT100	97
3.13	Schematic representation of development of TA29-full length antisense <i>nad3</i> and <i>atp9</i> constructs in pRT100	98
3.14	Schematic representation of development of T-ihp <i>nad3</i> and T-ihp <i>atp9</i> constructs in pRT100	99
4.1	Mitochondrial RNA of sunflower and safflower	117
4.2	PCR amplification of <i>atp6</i> with mitochondrial DNA of safflower and sunflower	117
4.3	RT-PCR for <i>atp6</i> with safflower mitochondrial RNA	120
4.4	Designing of primers for safflower <i>atp6</i> based on sunflower <i>atp6</i> gene sequence	120
4.5a & b	PCR with different combinations of primers for <i>atp6</i> of sunflower and safflower	120-121
4.6	RT-PCR with different primer combinations for <i>atp6</i> in safflower	121
4.7	PCR for <i>atp6</i> FIRI and FIIRI with safflower, sunflower and castor	121

Figure No.	Title	Page No.
4.8	Colony PCR for safflower T/A- <i>atp6</i> FIIIRI clones	123
4.9	Colony PCR for sunflower T/A- <i>atp6</i> FIRI clones	123
4.10	Restriction of safflower T/A- <i>atp6</i> FIIIRI and sunflower T/A- <i>atp6</i> FIRI with <i>SacI</i> to check the orientation	123
4.11	PCR for confirmation of <i>atp6</i> RACE product	125
4.12	Restriction of T/A-RACE clones with <i>HindIII</i> and <i>EcoRI</i>	125
4.13	Confirmation of sunflower T/A- <i>atp6</i> FIRI cDNA clones	125
4.14	PCR for <i>nad3</i> and <i>atp9</i> with mitochondrial DNA of safflower and sunflower	127
4.15	Confirmation of T/A- <i>nad3</i> and T/A- <i>atp9</i> genomic clones by restriction analysis with <i>HindIII</i> and <i>EcoRI</i>	127
4.16	RT-PCR for <i>nad3</i> and <i>atp9</i> of safflower	129
4.17	Confirmation of T/A- <i>nad3</i> cDNA clones	129
4.18	Confirmation of T/A- <i>atp9</i> cDNA clones	129
4.19	Comparison of genomic and cDNA sequences of <i>nad3</i> gene of safflower	131
4.20	Comparison of genomic and cDNA sequences of <i>atp9</i> gene of safflower	132
4.21	Editing in different cDNA clones of <i>nad3</i>	135
4.22	Editing in different cDNA clones of <i>atp9</i>	136
4.23	Comparison of <i>nad3</i> genomic and cDNA sequences of <i>C. tinctorius</i> with cDNA sequences of other species	137
4.24	Comparison of <i>atp9</i> genomic and cDNA sequences of <i>C. tinctorius</i> with cDNA sequences of other species	138
4.25	Comparison of the NAD3 protein deduced from the genomic and mRNA sequences of <i>C. tinctorius</i> with that of mRNA sequences of other species	140
4.26	Comparison of ATP9 protein deduced from the genomic and mRNA sequences of <i>C. tinctorius</i> with that of mRNA sequences of other species	140
4.27	Genomically encoded-unedited and cDNA encoded-edited NAD3 protein analysed by ProtScale using Kyte and Doolittle Scales for hydrophobicity	141

Figure No.	Title	Page No.
4.28	Genomically encoded-unedited and cDNA encoded-edited ATP9 protein analysed by ProtScale using Kyte and Doolittle Scales for Hydrophobicity	141
4.29	Diagrammatic representation of the transmembrane helices predicted by HMMTOP for unedited NAD3 and edited NAD3	142
4.30	Diagrammatic representation of the transmembrane helices predicted by HMMTOP for unedited ATP9 and edited ATP9	143
4.31a	Schematic diagram of primers used for serial PCR and the amplicons obtained for pCAMBIA1305.2-TCNN and pCAMBIA1305.2-TCAN	159
4.31b	Serial PCR for confirmation of pCAMBIA1305.2-TCNN and pCAMBIA1305.2-TCAN	159
4.32	Confirmation of pCAMBIA0390-TCNN and pCAMBIA0390-TCAN by restriction with <i>HindIII</i> and <i>EcoRI</i>	161
4.33	Confirmation of pBin-TCNN-Bar and pBin-TCAN-Bar	161
4.34a	Schematic representation for confirmation of orientation of <i>bar</i> cassette vs. male sterility gene cassette by restriction analysis	163
4.34b	Confirmation of orientation of pBin-TCNN-Bar and pBin-TCAN-Bar by restriction with <i>BamHI</i> and <i>SacI</i>	163
4.35a	Schematic representation of confirmation of p-SANP and p-SAAP by restriction with <i>KpnI</i> and <i>XhoI</i>	165
4.35b	Confirmation of p-SANP and p-SAAP by restriction with <i>KpnI</i> and <i>XhoI</i>	165
4.36	Restriction of p-SANP and p-SAAP with <i>HindIII</i>	167
4.37	Confirmation of pBin-SANP and pBin-SAAP by restriction with <i>HindIII</i>	167
4.38	Confirmation of T/A- <i>nad3</i> (ihp) and T/A- <i>atp9</i> (ihp) by restriction with <i>HindIII</i> and <i>EcoRI</i>	167
4.39a	Schematic representation of confirmation of the orientation of T/A- <i>nad3</i> (ihp) and T/A- <i>atp9</i> (ihp) clones by restriction with <i>BamHI</i>	169

Figure No.	Title	Page No.
4.39b	Confirmation of the orientation of T/A- <i>nad3</i> (ihp) and T/A- <i>atp9</i> (ihp) by restriction with <i>Bam</i> HI	169
4.40	Confirmation of pRT100-CI-antisense <i>nad3</i> and pRT100-CI-antisense <i>atp9</i> by restriction with <i>Hind</i> III	171
4.41	Confirmation of p-ihp <i>nad3</i> clones by restriction with <i>Hind</i> III	172
4.42	Confirmation of p-ihp <i>atp9</i> clones by restriction with <i>Hind</i> III	172
4.43	Confirmation of pBin-ihp <i>nad3</i> and pBin-ihp <i>atp9</i> clones by restriction with <i>Hind</i> III	172
4.44	Colony PCR for confirmation of presence of TA29 in p-TANP and p-TAAP	174
4.45	Confirmation of presence of TA29 in p-TANP and p-TAAP by restriction with <i>Hinc</i> II and <i>Xho</i> I	174
4.46	Colony PCR for confirmation of p-TA29 clones	174
4.47	Confirmation of p-TA29 clones by restriction with <i>Hinc</i> II and <i>Xho</i> I	176
4.48	Confirmation of p-T-ihp <i>nad3</i> and p-T-ihp <i>atp9</i> clones by restriction with <i>Xho</i> I	176
4.49a	Schematic diagram the confirmation of p-T-ihp <i>nad3</i> and p-T-ihp <i>atp9</i> clones by PCR using different primer combinations	178
4.49b	Confirmation of p-T-ihp <i>nad3</i> and p-T-ihp <i>atp9</i> clones by PCR	178
4.50	Colony PCR for confirmation of pBin-T-ihp <i>nad3</i> and pBin-T-ihp <i>atp9</i> clones (TAF + CIR primers) by restriction with <i>Pst</i> I	179
4.51	Confirmation of pBin-T-ihp <i>nad3</i> and pBin-T-ihp <i>atp9</i> Clones by restriction with <i>Pst</i> I	179
4.52a	Schematic diagram-confirmation of pBin-T-ihp <i>nad3</i> and pBin-T-ihp <i>atp9</i> clones by PCR using different primer combinations	180
4.52b	Confirmation of pBin-T-ihp <i>nad3</i> and pBin-T-ihp <i>atp9</i> clones by PCR	180

Figure No.	Title	Page No.
4.53	Restriction of p-Bin-TCNN-Bar and p-Bin-TCAN-Bar clones before mobilization into <i>Agrobacterium</i> strain LBA4404	182
4.54	Confirmation of LBA4404: p-Bin-TCNN-Bar and LBA4404: p-Bin-TCAN-Bar clones by restriction with <i>HindIII</i> and <i>EcoRI</i>	182
4.55	Confirmation of reverse-mated p-Bin-TCNN-Bar clones by restriction with <i>HindIII</i> and <i>EcoRI</i>	184
4.56	Confirmation of reverse-mated p-Bin-TCAN-Bar clones by restriction with <i>HindIII</i> and <i>EcoRI</i>	184
4.57	Confirmation of LBA4404: p-Bin-TCNN-Bar by PCR	185
4.58	Confirmation of pBin-SANP and pBin-SAAP clones by restriction with <i>HindIII</i> before mobilization into <i>Agrobacterium</i> strain LBA4404	185
4.59	Confirmation of reverse-mated pBin-SANP and pBin-SAAP clones by restriction with <i>HindIII</i>	186
4.60	Confirmation of reverse-mated pBin-SANP and pBin-SAAP clones by PCR	186
4.61	Confirmation of reverse-mated pBin-ihp <i>atp9</i> clones by restriction with <i>HindIII</i>	187
4.62	Confirmation of reverse-mated pBin-ihp <i>atp9</i> clones by restriction with <i>HindIII</i>	187
4.63	Confirmation of reverse-mated pBin-ihp <i>nad3</i> and pBin-ihp <i>atp9</i> clones by PCR	188
4.64	Confirmation of LBA4404: pBin-T-ihp <i>nad3</i> and LBA4404: pBin-T-ihp <i>atp9</i> clones by PCR	188
4.65	Confirmation of reverse-mated pBin-T-ihp <i>nad3</i> clones by PCR	190
4.66	Confirmation of reverse-mated pBin-T-ihp <i>atp9</i> clones by PCR	191
4.67	Confirmation of reverse-mated pBin-T-ihp <i>atp9</i> clones by PCR	192
4.68	Confirmation of orientation of reverse-mated pBin-T-ihp <i>nad3</i> and pBin-T-ihp <i>atp9</i> clones by restriction with <i>XbaI</i>	192
4.69	Confirmation of transgenic safflower TCNN-Bar plants by PCR	215

Figure No.	Title	Page No.
4.70	Confirmation of transgenic safflower TCAN-Bar plants by PCR	215
4.71	Confirmation of transgenic tobacco TCNN-Bar plants by PCR	216
4.72	Confirmation of transgenic tobacco TCAN-Bar plants by PCR	216
4.73	Confirmation of transgenic safflower T-ihp <i>nad3</i> plants by PCR	217
4.74	Confirmation of transgenic safflower T-ihp <i>atp9</i> plants by PCR	217
4.75	Confirmation of tobacco T-ihp <i>nad3</i> transgenics by PCR	218
4.76	Confirmation of transgenic tobacco T-ihp <i>atp9</i> plants by PCR	218

LIST OF PLATES

Plate No.	Title	Page No.
4.1	Response of the immature embryo explants on E5 medium – developing into whole seedlings	146
4.2	Immature embryos giving rise to somatic embryos (SE) along with multiple shoots on E3 medium	146
4.3	Explants showing embryogenic calli and embryo-like structures along with multiple shoots in E1 medium	149
4.4	Immature embryos cultured on SV1 medium showing formation of multiple shoots	149
4.5	Immature embryos cultured on SV2 medium showing formation of multiple shoots	154
4.6	Immature embryo axes transformed by <i>Agrobacterium</i> mediated method and cultured on SV1 medium	154
4.7	Immature cotyledons transformed by <i>Agrobacterium</i> mediated method and cultured on SV1 medium	155
4.8	Shoots induced from safflower explants transformed with TCNN-Bar construct	195
4.9	Enlarged view of shoots arising from transformed hypocotyl explants	195
4.10	Shoots induced from safflower explants transformed with TCAN-Bar construct	196
4.11	Response of transformed root explants (TCAN-Bar) on selection medium	196
4.12	Elongation of transgenic T-ihp <i>nad3</i> and T-ihp <i>atp9</i> safflower shoots	199
4.13	Root induction in safflower transgenic shoots	199
4.14	Hardening of safflower plants: Plants covered with plastic sheets upto 5-7 days after acclimatization (tissue culture lab) in vermiculite pots	202
4.15	Hardening of safflower plants: Plants covered with plastic sheets cut at the corners upto 10-14 days after acclimatization (tissue culture lab)	202
4.16	Hardening of safflower plants: Plastic sheets removed after 10-14 days after acclimatization and plants kept as such for 5-7 days (tissue culture lab)	202

Plate No.	Title	Page No.
4.17	Hardening of safflower plants: Plants initially in smaller vermiculite pots transferred to green house	203
4.18	Shoot induction in tobacco explants transformed with TCNN-Bar construct	304
4.19	Response of tobacco explants transformed with T-ihp <i>nad3</i> construct	204
4.20	Shoot induction in tobacco explants transformed with T-ihp <i>nad3</i> construct	205
4.21	Root induction and elongation of tobacco transgenic shoots	205
4.22	Hardening of tobacco transgenic plants	211
4.23	Hardening of tobacco transgenic plants: Plant in small vermiculite pots hardened in green house for 7-10 days	211
4.24	Hardening of tobacco transgenic plants: Plant transferred into larger soil filled pots	212
4.25	Safflower leaf assay for phosphinothricin sensitivity	212
4.26	Tobacco transgenic plants showing resistance to phosphinothricin and hygromycin	213
4.27	Control tobacco plants showing susceptible reaction to phosphinothricin and hygromycin	213
4.28	Flowering of hardened safflower transgenic shoots	221
4.29	Flowers of transgenic vs control untransformed safflower	221
4.30	Partial fertility in a safflower TCNN-Bar transgenic plant	223
4.31	Floral morphology of the partially fertile TCNN-Bar tobacco transgenic plants	224
4.32	Partial fertility in tobacco TCNN-Bar transgenic plants	225
4.33	Pollen germination in partial sterile transgenic tobacco plants	227
4.34	Capsules of TCNN-Bar transgenic tobacco plants	228
4.35	TCNN-Bar transgenic tobacco plant (99-1) showing severe flower drop	228

ACKNOWLEDGEMENTS

It is by the grace and blessings of the almighty that I have got an opportunity to take up and accomplish this challenging investigation. I thank him for endowing me with courage for keeping up my spirits and stamina, both mental and physical during the entire course of this study. I take pleasure in thanking all those who have provided motivation and encouragement for this work.

I am grateful to **Dr. D. M. Hegde**, Project Director, DOR, for extending all the facilities to carry out my research work and also for giving me the opportunity to learn from all scientific and technical endeavours of the institute. Whenever I have interacted with him, I have felt humbled and inspired by his simplicity, higher level of thinking, positive words and forthcoming cooperation.

I express my deep sense of gratitude and reverence to the chairman of my advisory committee **Dr. S. Sokka Reddy**, Professor (Biotechnology), ANGRAU, Rajendranagar, Hyderabad for his motivation, and constant encouragement through out the period of my study and also for placing me in a good working atmosphere under able guidance. With positive words, he has been instrumental in guiding me at difficult times, encouraging me when I did well, and pointing out whenever I erred, which helped me a lot in improving myself.

I do not have enough words to express the amount of reverence and gratitude I feel for the Co-chairman of my advisory committee, **Dr. V. Dinesh Kumar**, Sr. Scientist (Biotechnology), Directorate of Oilseeds Research (DOR), Rajendranagar, Hyderabad. His meticulous guidance, invaluable suggestions and constant encouragement has helped me not only at each step of this work but has also given me good confidence to take up challenging assignments in future. The frequent scientific discussions, friendly talks and arguments with him and other colleagues in the laboratory shall be etched in my mind for ever. I thank the lord for having given me this opportunity of working under such a nice and simple, but high thinking person.

I would like to place on records my gratitude to **Dr. M. Sujatha**, Sr. Scientist (cytogenetics), DOR, Rajendranagar, Hyderabad and member of my advisory committee for her wise counsel, co-operation, and constant evaluation at all stages of my work. Her suggestions have also been of valuable during edition of the thesis. I also thank **Dr. T.Y. Madhulety** and **Dr. P.V. Rao**, Professors of the Department of Plant Physiology and members of the advisory committee for their encouragement and cooperation during the period.

I wish to offer my genuine thanks to **Dr. T. Nageswar Rao**, **Dr. K. Hussain Sahib** (former Heads of the Department of Genetics and Plant Breeding, College of Agriculture, Rajendranagar, Hyderabad) and the present Professor and Head (Incharge), **Dr. Bramheshwar Rao** for having encouraged me and extended all possible help during the course of study. I also thank all the Professors, Associate Professors and Assistant Professors of our department including **Dr. V. Sudheer Kumar**, **Dr. Farzana Jabeen**, **Dr. M. Bharathi**, **Dr. Kuldeep Singh Dangi**, **Dr. D. Lokanada Reddy**, **Dr. N.A. Ansari** and **Dr. K. Radhika** for their support and encouragement during the period of study.

I extend my heartfelt gratitude to **Dr. A.R.G. Ranganatha**, Principal Scientist, Head CI, DOR, for providing for his constant encouragement and valuable suggestions throughout the period of study. I am also thankful to **Dr. S. N. Sudhakara Babu**, Senior scientist, DOR, and **Dr. (Mrs.) Lekha Dinesh Kumar**, Scientist C, CCMB, Hyderabad, for their forthcoming help and inspiring

words. I also thank **Dr. Nagaraj** and **Dr. Pathy** for their constant encouraging and optimistic words. I also thank **Dr. R. Meenakshi Sundaram**, Scientist (SS), DRR for his timely and forthcoming support. I express my sincere thanks to **Dr. S.R. Bhat**, Principal Scientist, NRCPB, NewDelhi, for his valuable suggestions in different aspects of this work and for his critical correction of the manuscript.

I am greatly indebted to my friends **K. Kalyani** and **M. Ashraf Ashfaq** for their forthcoming help, valuable suggestions, moral support and joyful company during the period of study. I affectionately acknowledge the help and support received from my friends and lab mates **Wankhade, Harinath, Narasimha, Radhika, Jyothi, Anusha and Somshekar**. I also thank **Madhu, Indu, Durga, Akshay, Raju, Kavitha, Amarendra, Srikanth, Venu, Pranavi, Saikumar, Shailaja, Tara, Shilpa, Kanaka, Lakshmi and Mani** for their support.

I am also thankful for the valuable help rendered by **Narasimha, Veeriah, Srikanth, Karna, Balamani, Bhagya, Shobha Rani** and others. I am very much pleased to express my gratitude to all my seniors and juniors in the lab as well as in the plant breeding department. I thank **Mr. B. V. Rao** for his support in taking photographs.

I feel deeply indebted to my parents **Shri. G. Narasimhan** and **Smt. Lakhmi Narasimhan** for their overwhelming moral support, affection, encouragement and patience. It is their constant, untiring efforts and constructive thoughts which have helped me in moulding my career and reach this juncture.

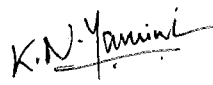
I do not find enough words but pleasant thoughts fill my heart for my husband **Dr. P. Rajendrakumar**. His love, patience, encouragement, moral support as well as scientific input have been indispensable and inspirational for me during the entire course of study.

My daughter, **Vandana's** cute smile and bright eyes full of zeal have provided me hope and confidence throughout. I express my warm feelings towards my beloved sweet sister **Nimmi**, her husband, **Suresh**, Little **Rishi**, Brother-in-law, **Viji** and **Parents-in-law** for their optimism and encouragement.

I am thankful to **Acharya N. G. Ranga Agricultural University**, for providing this opportunity to pursue my Doctorate degree in Genetics and Plant Breeding.

I also thank **Indian Council of Agricultural Research** for providing me funds through **Senior Research Fellowship**.

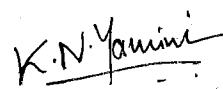
I convey my wholehearted thanks to all my well-wishers and friends seeking their forgiveness if I have not mentioned them by name.


K. N. YAMINI

DECLARATION

I, K. N. Yamini hereby declare that the thesis entitled **“Studies on development of transgenic male sterile and restorer lines in safflower (*Carthamus tinctorius* L.) using unedited mitochondrial gene(s)”** submitted to the Acharya N. G. Ranga Agricultural University for the degree of **Doctor of Philosophy in Agriculture** is the result of original work done by me. I also declare that any material contained in the thesis has not been published earlier in any manner.

Date : 24-10-07
Place : Hyderabad


(K. N. YAMINI)

Author : **K.N.Yamini**

Title of thesis : **Studies on development of transgenic male sterile and restorer lines in safflower (*Carthamus tinctorius* L.) using unedited mitochondrial gene(s)**

Degree : **Doctor of Philosophy in Agriculture**

Faculty : **Agriculture**

Discipline : **Genetics and Plant Breeding**

Major advisor : **Dr. S. Sokka Reddy**

University : **Acharya N. G. Ranga Agricultural University**

Year of Submission : **2007**

ABSTRACT

The present investigation was carried out with the aim of developing constructs and using them for the development of transgenic male sterile and restorer lines in safflower. The premise for the research project was that if proteins encoded by the unedited version of mitochondrial gene(s) are targeted into mitochondria, normal function of mitochondria would be disrupted and the expression of such genes, if spatially restricted to the tapetal cell layer in the anthers, would lead to ablation of tapetum resulting in male sterility.

In the first phase, studies were taken up with the aim of identifying suitable candidate genes for this approach. Three safflower mitochondrial genes viz., *atp6*, *atp9* and *nad3*, were studied. The results indicated the occurrence of substantial RNA editing in *nad3* and *atp9* transcripts and further analysis with the deduced protein sequences of edited and unedited transcripts indicated the importance of RNA editing in these genes. Based on these results, it was opined that both *u-nad3* and *u-atp9* genes could be appropriate candidate genes for induction of male sterility.

In the next phase, constructs for induction of transgenic male sterility and restoration of fertility were developed using the *u-nad3/u-atp9* genes. Towards the former, two constructs were developed which had the genomic (unedited) versions of these genes

under the control of TA29 promoter (for conferring specific expression in the tapetum) in-frame with *coxIV* presequence (for targeting the proteins into mitochondria) in the binary vector pCAMBIA0390. The *bar* gene cassette that confers resistance to the herbicide phosphinothricin, was cloned into these vectors resulting in two plant transformation vectors pBin-TCNN-Bar and pBin-TCAN-Bar.

Vectors for restoration of fertility based on post-transcriptional gene silencing (PTGS) approach were developed so that they could down regulate the expression of *u-nad3/u-atp9* genes thereby restoring fertility. Two strategies of PTGS viz., antisense RNA and intron interrupted hairpin RNA (ihp-RNA) were adopted for developing the gene constructs. The full-length antisense constructs of these *u-nad3* and *u-atp9* genes were developed under both 35S promoter (SANP and SAAP) and TA29 promoter (TANP and TAAP). Also the ihp-RNA vectors against both these genes under 35S promoter (p-ihp *nad3* and p-ihp *atp9*) and under TA29 promoter (p-T-ihp *nad3* and p-T-ihp *atp9*) were developed. Thus, a set of eight gene constructs were developed for fertility restoration and all these cassettes were cloned into the pCAMBIA1300 binary vector. All the confirmed binary vectors for both sterility induction and fertility restoration were mobilized into *Agrobacterium* strain LBA4404.

As reproducible regeneration and transformation protocols were not available in safflower, attempts were made under the present investigation towards regeneration of safflower shoots using immature embryos as explants. Culture of medium sized immature embryos (3-5 mm) of safflower on the three primary media combinations, MS + 3% sucrose + TDZ 1.0 mg/l + NAA 0.2 mg/l (SV₁), MS + 3% sucrose + TDZ 0.5 mg/l + NAA 0.2 mg/l (SV₂) or MS + 3% sucrose + TDZ 0.5 mg/l + NAA 1.0 mg/l (E₁) and then transfer of the responding explants into secondary medium MS + BA 0.2 mg/l gave rise to adventitious shoots in higher frequency.

The two constructs for induction of male sterility i.e., LBA4404: pBin-TCNN-Bar and LBA4404: pBin-TCAN-Bar as well as two constructs for restoration of fertility i.e., LBA4404: pBin-T-ihp *nad3* and LBA4404: pBin-T-ihp *atp9* were used to develop transgenic safflower and tobacco plants. All these transgenic plants were confirmed by PCR analysis for presence of the transgene and by leaf assays for sensitivity to phosphinothricin

or hygromycin (depending on the vector used). The plants and their flowers were observed for any morphological differences as well as for pollen fertility. Tissue culture derived shoots of safflower were lanky and they did not produce many flowers indicating the inherent problems of *de novo* regenerated shoots. Among the few transgenic safflower plants that reached flowering, three out of the six obtained with TCNN-Bar construct and one out of five obtained with TCAN-Bar construct, showed partial sterility (34.2%-69% and 32% pollen fertility respectively).

Of the tobacco transgenic plants that reached flowering, one plant out of thirty obtained with TCNN-Bar construct was sterile with less than 5% pollen taking up acetocarmine stain and these pollen grains exhibited abnormal morphology with none of them germinating on the pollen germination medium. The flowers of this plant were smaller in size and did not form capsules except when they were manually pollinated with pollen from control plants. Seven TCNN-Bar plants showed partial sterility (22% to 76.8% pollen fertility) as well as abnormalities in the pollen tube germination. Many of these partial sterile plants also had smaller sized flower buds with some of them exhibiting highly protruded stigma with anthers regressed below the level of stigma. The capsules formed on such partial sterile plants were smaller in size compared to those on the control and the fertile transgenic plants. All other plants obtained with male sterility constructs as well as fertility restoration constructs were completely fertile.

This study has provided the preliminary ground work and proof-of-concept that the genes *atp9* and *nad3* could be used as candidate genes for induction of male sterility in safflower and tobacco. The future line of work involves further molecular characterization of these transgenic plants along with development and characterization of more number of transgenic plants with these constructs to provide a strong footing to this approach. This could lead to development of a complete pollination control system, not only in safflower, but also in other crops.

LIST OF ABBREVIATIONS AND SYMBOLS

A1	:	Annigeri 1
bp	:	base pair
BSA	:	Bovine Serum Albumin
BAP	:	Benzyl Amino Purine
CaCl ₂	:	Calcium chloride
CAMBIA	:	Center for the Application of Molecular Biology to International Agriculture
CaMV	:	Cauliflower Mosaic Virus
CMS	:	Cytoplasmic Male Sterility
CAT	:	Chloramphenicol Acetyl Transferase
<i>coxIV</i>	:	cytochrome c oxidase subunit IV
CTAB	:	Cetyl Trimethyl Ammonium Bromide
DNA	:	Deoxy Ribo Nucleic Acid
dNTP	:	deoxy Nucleotide Tri-Phosphate
DOR	:	Directorate of Oilseeds Research
EDTA	:	Ethylene Diamine Tetra Acetic Acid
g	:	gram
GFP	:	Green Fluorescent Protein
Gln	:	Glutamine
GMOs	:	Genetically Modified Organisms
GMS	:	Genetic Male Sterility
<i>gus</i>	:	glucuronidase
h	:	hours
HCl	:	Hydrochloric acid
HEPES	:	N- (2-Hydroxyethyl) piperazine-1-ethanesulfonic acid
HgCl ₂	:	Mercuric Chloride
<i>hpt</i>	:	hygromycin phosphate transferase
IBA	:	Indole Butyric Acid

IPTG	:	Isopropyl- β -D-thiogalactopyranoside
IVS	:	Intervening sequences
kDa	:	kilo Dalton
KCl	:	Potassium Chloride
<i>lacZ</i>	:	β -galactosidase
LA	:	Luria Agar
LB	:	Luria Broth
Leu	:	Leucine
<i>luc</i>	:	luciferase
M	:	Molar
MCS	:	Multiple Cloning Site
MgCl ₂	:	Magnesium Chloride
min	:	minutes
ml	:	millilitre
mM	:	millimolar
MS	:	Murashige and Skoog
MUG	:	4-methyl umbelliferyl β -D glucuronide
NAA	:	Naphthalene Acetic Acid
NaCl	:	Sodium Chloride
NaOH	:	Sodium hydroxide
ng	:	nano gram
<i>nos</i>	:	nopaline synthase
<i>ocs</i>	:	octopine synthase
<i>orf</i>	:	open reading frame
OD	:	Optical Density
O/N	:	Over night
PCR	:	Polymerase chain reaction
PET1	:	Petiolaris 1
pH	:	pH
PUFA	:	Polyunsaturated fatty acids
RES	:	Restriction Enzyme Sites

rpm	:	revolutions per minute
SDS	:	Sodium Dodecyl Sulphate
TA29	:	Tobacco Anther 29
<i>Taq</i>	:	<i>Thermophilus aquaticus</i>
T ₁₀ E ₁	:	10 mM of Tris.Cl, 1 mM of EDTA
T ₁₀	:	10 mM of Tris.Cl
TAE	:	Tris.Cl, Acetic Acid, EDTA
T-DNA	:	Transferred DNA
TDZ	:	Thiadiazuron
TE	:	Tris.Cl, EDTA
T _m	:	Temperature of melting
tRNA	:	transfer Ribonucleic Acid
Tris.Cl	:	Tris (hydroxy methyl) aminomethane hydro chloride
U	:	units
V	:	volts
X-Gal	:	5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside
X-Gluc	:	5-bromo-4-chloro-3-indolyl- β -D-glucuronide
°C	:	Degree Celsius
μ g	:	micro gram
μ l	:	microlitre
μ M	:	micro Molar
μ F	:	micro Faraday

CHAPTER I

INTRODUCTION

CHAPTER I

INTRODUCTION

Safflower (*Carthamus tinctorius* L.) is an important oilseed crop of India and its edible oil with the presence of very high levels of polyunsaturated fatty acids (PUFA), is considered to be one of the best oils from the health point of view. India ranks first in the world with a share of 47% of the area (380,000 hectares) and 27% of the production (210,000 tonnes) of safflower. Under the present edible oil scenario, increasing the productivity of this crop is of great significance. Hybrid safflower offers immense potential for exploitation of substantial heterosis reported to the extent of 227% for seed yield and 80% for oil production per unit area (Reddy, 1997). But this is not an easy task as safflower is predominantly a self-pollinated crop and production of true hybrid seeds without artificial pollination control is not economically viable.

Hybrid vigour, or heterosis, describes the increased vigour of F_1 plants when compared to either of the parents that are dissimilar in one or more inherited characters. The potential use of hybrid vigour in plants is always tantalizing to the breeder because it promises a new plateau of productivity. To meet the demand of food for the burgeoning population, the vigorous adoption of hybrid seed technology is essential. The most important step towards that direction is the development of a method of utilizing heterosis economically under commercial conditions. In several crops, the performance and utility of hybrids has been well demonstrated and is already being widely exploited.

Genetic male sterility (GMS) system has been utilized in the production of hybrids, but it is hampered in safflower by inherent problems like maintenance of male sterile lines (segregation into 50% sterile and 50% fertile lines), absence of a good seedling marker, spiny nature of the crop *etc.* Cytoplasmic Genetic Male Sterility (CGMS) which results from the interaction among the cytoplasmic (mitochondrial) and nuclear genes is a viable alternative to GMS and has been adopted for hybrid production in safflower by a private company in United States (Hill, 1989). But this system could not be utilized in India since the plants exhibited lack of fertility restoration (Singh *et al.*, 2000). Such inherent bottlenecks faced during the development of male sterile lines through conventional breeding demands for biotechnological intervention to effect pollination control in safflower. Genetic engineering offers a precise tool to introduce specific traits into plants. Development of male sterile lines through transgenic approach has gained popularity in recent times. Several strategies have been used to produce transgenic male sterile plants by expressing degradative enzymes, inhibition of particular enzymes by antisense strategy or expression of hormone encoding genes, by expressing genes known to induce male sterility in a heterologous system *etc.* In most of these cases, cell lines or tissues involved in normal pollen formation and/or release have been ablated so that the microsporogenesis is affected. It has also been established that if mitochondrial function is impaired, especially in the tapetal cell layer of anthers that have a higher requirement of energy during pollen formation and development, it leads to male sterility.

RNA editing in plant mitochondria is a post-transcriptional process involving mainly the partial change of C residues into U. These C to U changes lead to the synthesis of proteins with an amino acid sequence different to that predicted from the gene. The

hypothesis that RNA editing leads to functional proteins in mitochondria has already been tested and confirmed. When unedited *atp9* from wheat was expressed in tobacco as well as *Arabidopsis* and the resultant protein was targeted into mitochondria, it resulted in male sterile phenotype (Hernould *et al.*, 1993; Gomez-Casati *et al.*, 2002). When these plants were crossed with the transgenics carrying antisense *u-atp9* gene, the fertility was restored in F₁ plants (Hernould *et al.*, 1998). A private company Avesthagen Graine, based in Bangalore, has also exploited this concept of using unedited mitochondrial genes to induce male sterility successfully in rice (http://www.avesthagen.com/docs/newsletters/jan_feb_2005.pdf).

The present investigation envisaged identification of mitochondrial genes with substantial RNA editing in safflower and use of the unedited (genomic) sequences of these genes to develop transgenic male sterile and restorer lines. To limit the expression of the non-functional protein (resulting from unedited version of the gene) in tapetal tissues of the anthers, a tapetum specific promoter, TA29 from tobacco was used. A pre-sequence, *coxIV* from yeast was used to target the unedited protein product into the mitochondria, which is its site of action. Both TA29 and *coxIV* sequences, have already been used successfully in many crops. The selected genes along with the other components in appropriate orientation were used to develop sense (for induction of male sterility) and antisense/RNAi (RNA interference) based constructs (for restoration of fertility). It is hypothesized that the sense construct when transferred into safflower, will lead to impairment of mitochondrial function and result in male sterility. Also, the antisense/RNAi based constructs targeted against *u-nad3/u-atp9* would be used to produce transgenic plants, which would be used as restorers

of fertility. Tobacco was used as a control system to facilitate easier validation of the developed constructs. The work was carried out with the following objectives:

1. Identification of appropriate mitochondrial gene(s) for induction of transgenic male sterility in safflower by RNA editing studies.
2. Standardizing the shoot regeneration in safflower through embryogenic pathway.
3. Cloning of the identified mitochondrial gene(s) in appropriate orientation to develop the sense and antisense constructs.
4. *Agrobacterium*-mediated transformation of safflower and tobacco with the developed constructs.
5. Confirmation of the developed safflower and tobacco transgenics.

CHAPTER II

REVIEW OF LITERATURE

CHAPTER II

REVIEW OF LITERATURE

Safflower (*Carthamus tinctorius* L.), an important oilseed crop belonging to the family Asteraceae, has been grown for centuries in India. It is described as “kusumbha” in ancient Sanskrit literature (Chavan, 1961), from which other names like kusum, karrad, kusuma, kusumba *etc.*, have been derived. Safflower has been grown since ancient times for its orange red dye extracted from its florets which was largely used for colouring purposes in food and textile industry. With the availability of cheaper synthetic dyestuffs, it is now grown for its much valued edible oil.

Safflower produces oil rich in polyunsaturated fatty acids (78% linoleic acid) which play an important role in reducing blood cholesterol level and hence is considered as a healthy cooking medium. The oil is used in preparation of infant foods, liquid nutrition formulations and also for illuminating and as hair oil.

All parts of the safflower plant find useful applications especially in herbal medicine for the treatment of physical disorders. The seeds are parched and eaten whereas the florets and tender leaves are used as vegetable in traditional dishes. Flowers are not only sold as substitutes for saffron, but also used in preparation of cosmetics. Safflower oil has industrial utility in preparation of paints, varnishes, surface coatings, alkyl resins, water-proof clothes *etc.* It is an effective non-allergenic dispersing agent for injectable medications and is also used for healing sores and in rheumatism. The safflower plant also makes acceptable livestock forage. Uncorticated safflower cake is used as manure while decorticated safflower cake is fed to ruminants and monogastric animals. Safflower has also gained importance as a poultry food in recent years.

Safflower is believed to have originated in an area bound by Eastern Mediterranean and Persian Gulf. *C. oxycanthus* Bieb. and *C. persicus* Willd. are considered the parental species of cultivated safflower. *C. palaestinus* Eig. is the progenitor for both the parental species. All the four species have the genome constitution BB and $2n=24$ chromosomes (Ashri and Knowles, 1960).

The world safflower production has shown wide fluctuations with the current production being 0.947 million tonnes from an area of 1.174 million hectares. With an area of 380 thousand hectares accounting for 47% and production of 210 thousand tonnes accounting for 27%, India ranks first in the world followed by Mexico, USA, Ethiopia, Australia and Europe. Among the states in India, Maharashtra is the largest producer of safflower (63%), followed by Karnataka, Andhra Pradesh, Orissa, Madhya Pradesh and Bihar. Increasing the productivity of this crop is of great importance for our country especially under the present scenario of the domestic edible oil production meeting only about 50-55% of the inland requirement and the rest being imported (Hegde, 2006).

This review provides a general background regarding the research topic and then enumerates the relevant information pertaining to the research topic. Before going into the need for hybrids in safflower and the work done in this area, male sterility systems in this crop shall be reviewed.

2.1 Male sterility systems in safflower

Hybrid safflower offers immense potential for exploitation of substantial heterosis reported to an extent of 227% for seed yield and 80% for oil production per unit area (Reddy, 1997). Hybrids produced from different varieties of the same species (F_1 hybrids)

are often more vigorous than either of the parents and hence they are preferred by the growers. But safflower being a self-pollinated crop, hybrid seed production is not an easy task and production of true hybrid seeds without artificial pollination control is economically unviable. Cross-pollination, mainly through bees, to the extent of 28% has been reported in safflower (Kadam and Patnakar, 1942). A simple method of achieving cross-pollination is through the utilization of male sterility systems, which have been successfully exploited in many crops.

Male sterility of some form has been found in many crops, and breeders are always on the alert for identifying such plants among their selections. The occurrence of male sterility was first documented in 1763 when Kolreuter observed anther abortion within species and in interspecific hybrids. Ways to induce male sterility like manual emasculation and chemical removal of pollen using male specific gametocides that disrupt pollen development or release, have been employed, but have several associated problems (cost, labour *etc.*) that make them unpopular techniques. Two types of male sterility have become economically significant in safflower: genetic male sterility (GMS) and cytoplasmic genetic male sterility (CGMS).

2.1.1 Genetic male sterility

GMS involves failure of pollen formation due to one or more (dominant or recessive) nuclear genes. GSM originating through spontaneous mutation is a common phenomenon in nature and is of wide occurrence in flowering plants. This system has limited utility in hybrid seed production because it is not possible to fix the allele for male sterility and only a maximum of 50% male sterile plants can be produced. The remaining 50% constitute fertile

heterozygous plants that have to be essentially rogued out of the seed production plots, which add to the labour and cost of seed production. Cases of GMS have been reported in safflower. Deshpande (1940) observed a sterile mutant having thick, twisted and unbranched stem with a solitary capitula which did not open and failed to set seed. Subsequently, there have been reports of GMS in safflower controlled by recessive (Richharia, 1941; Heaton and Knowles, 1980; Ramachandram and Sujatha, 1991; Singh, 1996; Singh, 1997, Anjani, 1997) and dominant genes (Joshi *et al.*, 1983; Jambhale and Nerkar, 1983). Each of these male sterility sources segregated in the ratio of 1:1 for male sterile and male fertile plants. The male sterile plants in GMS system can be identified when the first floret protrudes out. The capsules in male sterile plants can be distinguished even from a distance by their distinct pinched appearance. However, identification of seedling markers will enhance the efficiency of GMS lines in hybrid seed production.

A limited success has been achieved in hybrid seed production of safflower through the development of GMS lines. Experimental safflower hybrids developed using GMS system have revealed a genetic potential to give 25-38 quintals of seed yield per hectare (Reddy, 1997), giving credence to the importance of hybrids in breaking the yield barriers. The details of commercial GMS based safflower hybrids in India are reviewed in section 2.2. The associated problems of identification and maintenance of the male sterile individuals in such system makes the entire venture of seed production tedious and uneconomical. The problem is further accentuated in safflower because of the spiny nature of the crop. Therefore, alternative pollination control systems are urgently needed to realize heterotic advantage in safflower. One viable alternative is the development of CGMS system.

2.1.2 Cytoplasmic genetic male sterility

CGMS results from the interaction among the cytoplasmic (mitochondrial) and nuclear genes. The terms CMS (cytoplasmic male sterility) and CGMS are often used interchangeably. CMS has been reported in more than 200 plant species (Kaul, 1988). Since the offsprings inherit the cytoplasm of the female parent, the cross involving the CMS plant as female leads to completely male sterile progeny. Typically, one or more nuclear genes, often called the fertility restorer genes can compensate for the cytoplasmic defect(s) and normal pollen formation occurs. CGMS has found large-scale applications for plant breeders and seed companies in the production of hybrid seeds in several crops. But CGMS has some limitations such as undesirable side effects, imperfect fertility restoration, effect of modifying genes, environmental effects *etc.* CGMS lines are usually obtained by creating alloplasmic genotypes through wide hybridization. However such lines show several abnormalities both in floral parts as well as in vegetative parts usually leading to yield penalty. Apart from this, identification of fertility restorer gene(s) for such alloplasmic CGMS lines could be time consuming and many times eluding the efforts of breeders.

The possibility of development and exploitation of CGMS for producing hybrids in safflower was first demonstrated in United States (Hill, 1989). However, all the hybrids based on this system which were evaluated during 1998-99 at several locations in India exhibited a lack of fertility restoration (Singh *et al.*, 2000). Therefore, this male sterility system available in the United States could not be exploited commercially in India. These limitations in natural systems, plus the substantial economic considerations, have necessitated the need for concerted efforts by biotechnologists to develop novel strategies for male sterility systems using genetic engineering.

2.2 Safflower hybrids in India

In India, safflower hybrids based on UC148, a GMS line released from University of California at Davis are available (Deshmukh *et al.*, 1989). This system is labour intensive as the plants have to be checked for pollen shedders throughout the flowering season and is not feasible. The first safflower hybrid from the public sector in India is DSH-129, developed at Directorate of Oilseeds Research (DOR), Hyderabad involving recessive GMS system (Anjani, 1998). It is a cross between a GMS line MS-9 (O) and the high yielding variety A1. Another hybrid MKH-11, was developed using dominant GMS system by Maharashtra Hybrids Seeds Company Ltd. (MAHYCO) from a cross between MS5008 and NMK3480. These two were the first safflower hybrids released for commercial cultivation in India in 1997. Subsequently, PH-6 (NH-1), the GMS based first non-spiny hybrid developed from MMS X C2829-5-39-6 cross by Nimbkar Agricultural Research Institute (NARI) was released in the year 2001. More recently, another hybrid NARI-H-15 has been released in 2005. Both these hybrids were developed using GMS system. Through hybrid development, an increase of over 30% of seed yield as well as oil yield was achieved over the national check variety A-1 (Hegde, 2006).

Recently, a CMS-based hybrid, MRSA-521, has been developed by MAHYCO and has been identified for release in India (Pandey *et al.*, 2007). The stable performance of hybrids under various abiotic stress conditions indicates that hybrid safflower is a boon to farmers to overcome abiotic stress conditions occurring frequently during the crop growth period. The above mentioned hybrids have been released for commercial cultivation in India, but the basic problems concerning GMS-based safflower hybrids are the increased cost, time and efforts for maintenance of male sterile lines, spiny nature of safflower plant

etc. due to which varieties are more preferred by the farmers. Problems associated with the development as well as the exploitation of CMS and GMS systems necessitate the need for the development of alternate and viable methods through biotechnological approaches. Any successful attempt in this direction in safflower would be of great significance for increasing the productivity of this crop.

2.3 Molecular basis of CMS

CMS plants have defective male floral organs but usually have normal female floral organs and exhibit no defects in vegetative parts. CMS phenotypes range from the inability to produce fertile pollen in otherwise normal flowers to drastically altered flower architecture. Altered flower phenotype or defects in pollen formation are presumed to be secondary effects of the mitochondrial mutation. The primary defect may be a reduction in the efficiency of respiration or the impairment of anther mitochondrial function. Typically, one or more nuclear genes together with mitochondria bearing the CMS mutation, restore male fertility and are therefore termed fertility restorer genes (or restorer genes) (Touzet and Budar, 2004). At least some restorer genes reflect a co-evolution of mitochondrial and nuclear genomes. Other nuclear genes (the so-called maintainer genes) allow for the expression of the CMS phenotype and stabilize it (Kaul, 1988). Thus, expression of CMS is the result of a subtle interaction between mitochondrial and nuclear genes. Consequently, CMS is often observed in the offspring of crosses between different species, or after fusion between protoplasts of different species/genera followed by regeneration of hybrid or 'cybrid' plants, *i.e.* when mitochondrial and nuclear genomes that have not coevolved, are brought together (Kaul, 1988; Zubko, 2004).

CMS has been genetically well-studied and plant breeders as well as seed companies have been utilizing CMS since 1960s for hybrid seed production. Maize was the first crop for which CMS was extensively used in hybrid seed production. At the end of the 1960s, more than 80% of hybrid corn seeds were produced in USA by exploiting CMS caused by the T (Texas) cytoplasm. But a fungal disease specifically affecting maize plants with the T-cytoplasm resulted in severe yield losses. This 'T-maize disaster' led to investigations of the molecular mechanisms of CMS (Levings, 1993). Investigations regarding the mechanism of CMS in different crops have identified the actual changes in the mitochondrial genomes that have effected the sterility. Invariably all these mutations have been mapped onto the mitochondrial genome. Detailed molecular analyses have led to the identification of gene(s) responsible for CMS. Table 2.1 provides a list of the genes so far implicated in CMS in different crops. In most of the cases, sterility has been ascribed to the creation of new *orfs* (open reading frames) due to rearrangements in the mitochondrial genomes. This has been established by inducing sterility in plants by expressing *orfs* from heterologous systems.

2.4 Transgenic male sterility

Naturally occurring CMS mutations and/or appropriate fertility restoration genes have not been found in all crop species. Hence, problems with GMS and CMS systems have, of late, directed research efforts towards use of recombinant DNA techniques for developing efficient male sterility systems. Most of these investigations, except for recent efforts to engineer CMS via chloroplast transformation (Chase, 2006), are towards engineering nuclear male sterility. One strategy is to directly prevent or impair pollen formation and/or development (by targeting pollen grains or tapetal tissue) and the other is to impair or

Table 2.1 Examples of mitochondrial sequences/genes associated with CMS

Plant	orf	Flanking genes	Mitochondrial genes/gene portions involved	References
Bean	<i>pvs-orf239</i>	-	<i>atp1</i> , 3'-regions of <i>cob</i> , <i>orf239</i>	Mackenzie and Chase, 1990
Petunia	<i>pcf</i>	<i>atp9</i>	<i>atp9</i> , <i>coxII</i> , <i>urfS</i> , <i>orf143</i> , and <i>rps12</i> , <i>nad3</i> (co-transcribed)	Young and Hanson, 1987; Hanson <i>et al.</i> , 1999
Sunflower	<i>orf522</i>	<i>atp1</i>	<i>atp1</i> , <i>atp8</i>	Horn <i>et al.</i> , 1991; Moneger <i>et al.</i> , 1994; Sabar <i>et al.</i> , 2003
Sorghum	<i>orf107</i>	<i>atp9</i>	<i>atp9</i>	Tang <i>et al.</i> , 1996
Radish <i>kosena</i>	<i>orf125</i>	<i>trnfM/atp8</i>	No similarities	Iwabuchi <i>et al.</i> , 1999
Brassica <i>ogura</i>	<i>orf138</i>	<i>trnfM/atp8</i>	No similarities	Bonhomme <i>et al.</i> , 1991
Brassica <i>pol</i>	<i>orf224</i>	<i>atp8/atp6</i>	<i>atp8</i> , <i>rps3</i> , <i>orf224</i> and <i>atp6</i> (co-transcribed)	Singh and Brown, 1991; Handa <i>et al.</i> , 1995
Brassica <i>nap</i>	<i>orf222</i>		<i>atp8</i> and <i>nad5c</i> (exon c), <i>orf139</i> (co-transcribed)	L'-Homme <i>et al.</i> , 1997
Rice	<i>orf79</i>	<i>atp6</i>	<i>atp6</i> , <i>coxI</i>	Akagi <i>et al.</i> , 1994
T-maize	<i>urf-13</i>	<i>atp6/atp4</i>	<i>rrn26</i> , <i>atp4</i> , tRNA ^{Arg}	Dewey <i>et al.</i> , 1986

Abbreviations: *orf*, open reading frame; *urf*, unidentified open reading frame; *nad*, subunit of complex I; *cob*, *coxI*, *coxII*, subunits of cytochrome oxidase; the subunits of the ATP-synthetase F₀/F₁ components are indicated as *atp1* (also designated as *atpA*), *atp4* (former *orf25* or *orf221*, gene product is similar to the beta-subunit of the F₀ stalk; *atp6*, *atp8* (former *orfB*); *rrn26*, 26S ribosomal rRNA; *rps3*, ribosomal protein; *trnfM*, fMet-tRNA; the functions of *orf139* and *urfS* are not known.

disrupt mitochondrial functioning leading to severe energy constraints that in-turn affect pollen grains or the tapetal tissues. Since in all these approaches, the main thrust is to affect male gametophyte development, it is appropriate to discuss the different processes involved in male gametogenesis before reviewing the various strategies for transgenic male sterility.

2.4.1 Male gametophyte development

The male reproductive organs, the stamens, consist of an anther which is the site of pollen development and in most of the species, a stalk like filament transmits water and nutrients to the anther and positions it to aid pollen dispersal. Within the anther, male sporogenous cells differentiate and undergo meiosis to produce microspores, which give rise to pollen grains, whereas other cell types contribute to pollen maturation, protection or release. Pollen development involves an array of extraordinary events, including cell division and differentiation independent of a conventional meristem, the transition from sporophytic to gametophytic generation, modifications of cell division to produce structures that are unusual in plant development, including coenocytic tissues (the tapetum and the microsporocyte mass), and subsequently free cells (microspores) that give rise to self-contained units for genome dispersal (pollen grains).

The identity of floral organ primordia is controlled by three classes (termed A, B, and C) of homeotic selector genes with overlapping areas of activity; stamens develop in the third whorl, where both B and C genes are expressed. It has been discovered that additional transcription factors, encoded by *SEPALLATA* (*SEP*) genes in *Arabidopsis*, are required to confer full activity on the homeotic genes in stamens (as well as petals and carpels). The ABC model was based on genetic studies in *Arabidopsis thaliana* and *Antirrhinum majus*.

(Coen and Meyerowitz, 1991). The B class genes are *APETALA3* (*AP3*) and *PISTILLATA* (*PI*) in *Arabidopsis* and *DEFICIENS* (*DEF*) and *GLOBOSA* (*GLO*) in *Antirrhinum*; the C genes are *AGAMOUS* (*AG*) and *PLENA*, respectively. Mutations in any one of the B or C genes result in homeotic conversion of the third-whorl organs to a different type. Loss of B function causes transformation of stamens to carpels, loss of C function converts stamens to petals, and loss of both transforms stamens to sepals.

2.4.1.1 Anther development

Anther development initiates with the emergence of the stamen primordia in the third whorl of the floral meristem and concludes with the release of pollen grains at dehiscence (Goldberg *et al.* 1993). This process, including the dehiscence program, occurs in two phases. In phase 1, differentiation events establish the four locules and the stereotyped pattern of cell types that originate from the L1, L2, and L3 cell layers of the primordium are present in the mature anther. These include stomium and circular cell cluster formation in the notch region of the expanding anther. In addition, microspore mother cells within the locules undergo meiosis to generate haploid microspores. During phase 2, filament elongation occurs, anther enlargement takes place, pollen grains differentiate from microspores in each locule, fibrous bands appear in endothelial and connective cells, and cell degeneration events within the connective, circular cell cluster, and stomium lead to pollen release at flower opening (Goldberg *et al.*, 1993; Beals and Goldberg, 1997; Sanders *et al.*, 2005).

2.4.1.2 Pollen development

As the anther develops, it assumes a four lobed appearance and four groups of archesporial cells corresponding to the four microsporangia are observed. These cells divide to form the outer parietal layer and the inner sporogeneous tissue. The former divide and give rise to the endothecium, middle layers and the tapetum while the latter undergoes a few mitotic divisions to give rise to microspore mother cell. The microspore mother cells and the tapetum (which is the source of nourishment for the developing pollen) are sites of intense metabolic activity during meiosis. Each microspore mother cell (diploid) divides by meiosis to produce four microspores (haploid). This cluster of four microspores is called a tetrad of microspores. Callose deposition is initiated at the onset of meiosis (around individual microspore mother cells) and continues through meiosis. Isolation of microspores (within a tetrad) by a callose wall seems to be essential for normal development of pollen. Later, the callose wall around microspores is degraded by the activation of callase (β -1,3 glucanase) and the microspores are released into the anther locule. Each microspore differentiates into a pollen grain by developing a heavy thick and sculptured wall around itself. Division of the microspore is a major morphogenetic event after its release from the tetrads. Each microspore divides once by mitosis to form either a 2-celled microspore or a binucleated (forming a vegetative and a generative cell) microspore depending upon the plant species (reviewed by Shivanna, 2002).

2.4.1.3 Role of tapetum

Most of the strategies (that try to mimic natural CMS) for induction of male sterility attempt to disrupt the activity of tapetum. This is because

2.4.1.2 Pollen development

As the anther develops, it assumes a four lobed appearance and four groups of archesporial cells corresponding to the four microsporangia are observed. These cells divide to form the outer parietal layer and the inner sporogeneous tissue. The former divide and give rise to the endothecium, middle layers and the tapetum while the latter undergoes a few mitotic divisions to give rise to microspore mother cell. The microspore mother cells and the tapetum (which is the source of nourishment for the developing pollen) are sites of intense metabolic activity during meiosis. Each microspore mother cell (diploid) divides by meiosis to produce four microspores (haploid). This cluster of four microspores is called a tetrad of microspores. Callose deposition is initiated at the onset of meiosis (around individual microspore mother cells) and continues through meiosis. Isolation of microspores (within a tetrad) by a callose wall seems to be essential for normal development of pollen. Later, the callose wall around microspores is degraded by the activation of callase (β -1,3 glucanase) and the microspores are released into the anther locule. Each microspore differentiates into a pollen grain by developing a heavy thick and sculptured wall around itself. Division of the microspore is a major morphogenetic event after its release from the tetrads. Each microspore divides once by mitosis to form either a 2-celled microspore or a binucleated (forming a vegetative and a generative cell) microspore depending upon the plant species (reviewed by Shivanna, 2002).

2.4.1.3 Role of tapetum

Most of the strategies (that try to mimic natural CMS) for induction of male sterility attempt to disrupt the activity of tapetum. This is because pollen sterility

(nuclear/cytoplasmic/environmental) is invariably associated with tapetal abnormality. Direct and indirect evidences suggest that the tapetum plays a crucial role in pollen development. Tapetum is the nurse tissue for developing pollen as it facilitates the passage of nutrients into the anther locules. Tapetum also releases the enzyme callase, which is required for breakdown of the callose wall surrounding the microspores so that they are released from the tetrads. Tapetal activation of callase at the right stage is very important for the normal development of pollen. Tapetum is also considered to provide some precursors (like sporopollenin, tryphine *etc.*) that are important for exine formation (outer protective coat for pollen). (reviewed by Shivanna, 2002).

2.4.1.4 Genes involved in anther and pollen development

The network of gene function is expected to be very complicated in male gametophyte development, with an estimate of more than 50% of the genes encoded by a genome being active in the male gametophyte. Programmed cell death also plays an important role in the anther dehiscence with a series of progressive degenerations of different cell types (Sanders *et al.*, 2005). Stamen development and gene expression have been studied and reviewed in the past (Scott *et al.*, 1991; Goldberg *et al.*, 1993; Irish 1999) and more recently (Scott *et al.*, 2004; Wilson and Yang, 2004; Sanders *et al.*, 2005). Several of the genes involved, have been identified by using either transcriptional profiling methods or by screening for mutations that affect the stamens in many crops (reviewed by Scott *et al.*, 2004). For example, the genes involved in stamen development and function include APETALA3 (*AP3* involved in the specification of petals and stamens), *tap1* (expressed in tapetum and encoding a putative secreted protein), *fil1* (filamentous flower1-expressed

mainly in stamen filaments and petal bases and encoding a candidate cell-wall protein), *nap* (NAC-LIKE, ACTIVATED BY *AP3/PI*, expressed in stamens and petals, involved in the transition between cell division and expansion), *TPD1* (TAPETUM DETERMINANT 1 gene that is required for the specialization of tapetal cells in the *Arabidopsis* anther) etc. Some of the genes important for pollen fertility include DETERMINATE INFERTILE1 (*DIF1/SYN1*, encoding a cohesin required for chromosome segregation), MALE STERILITY2 (*MS2* encoding a predicted fatty acyl reductase with a potential role in pollen wall formation) and *MS1* and ABORTED MICROSPORES, both candidate transcriptional factors expressed in the tapetum and possibly in microspores (reviewed by Scott *et al.*, 2004). Though footholds have been established in some areas like patterning of the microsporangium, regulation of meiosis, and anther dehiscence, several other processes of the male gametophyte development need to be understood completely for elucidation of the entire developmental pathway.

2.5 Requirements for induction of transgenic male sterility

The following components/issues are to be considered carefully before working towards development of transgenic male sterility-fertility restoration systems.

a) Genes: A careful selection of gene(s) used for development of appropriate constructs that could be deployed for inducing male sterility is the most important step. The target gene(s) should preferably not have any pleiotropic effects so that only the intended change is brought about without any side effects. One should avoid expressing cytotoxic genes of serious nature (*eg.* RIPs like ricin, diphtheria toxin, *etc.*) so that it will not warrant any controversies from the environment protection groups. In this regard, genes of plant origin

with no known toxicity to either animals or plants should be preferred to increase the social acceptability of the resultant transgenic plants. Genes encoding cytotoxic proteins (*barnase*, RNase-T1, DsRIP, DT-A), genes that encode proteins which can convert non-toxic substrates into cytotoxic compounds (*argE*), genes involved in metabolic processes (modified PR-b-1,3-glucanase, *nin88*), antisense or RNAi versions of genes important for male gametophyte development (*chs*, *bcp1*, PDH_E1 Alpha1, SnRK1, CYP86MF, *susy*), *orfs* encoding proteins that are implicated in CMS systems (*orf239*, *orf79*, *orf138*) as well as unedited versions of important mitochondrial genes (*u-atp9*, *u-nad9*) have been used successfully for inducing transgenic male sterility (refer to table 2.2).

b) Promoters: It is well established that the transgenes introduced into plants, should be under the most appropriate promoter to regulate the expression of the introduced gene(s) spatially and temporally to suit the purpose. In the case of transgenic male sterility, the expression of the gene(s) should preferably be restricted to only the specific cell types within the anther where its role is implicated. Therefore, it is very essential to identify and choose promoters that will drive the expression of selected genes in the desired tissues or cell types. Seminal work of Goldberg's group (Koltunow *et al.*, 1990; Beals and Goldberg, 1997) has led to identification of such promoters, which are being used in different approaches. Apart from these, many promoters with specific activities in pollen grains or meiotic cells have been used for induction of male sterility. Also, it should be kept in mind that if pollen specific promoters are used, it basically leads to sterility of pollen grains carrying the gene and thus can only lead to gametophytic male sterility that is difficult to adopt for exploitation of hybrid vigour. Instead, when promoters that are active in sporophytic tissues are used for targeting the tissues in anthers like tapetum, stomium,

circular cell complex *etc.*, then completely male sterile plants are expected. Different promoters used in the various approaches for inducing male sterility are constitutive (*e.g.* CaMV35S, ubiquitin, *sp*), pollen specific (*e.g.* PS1, Lat52, *sp*), anther specific (*e.g.* TA29, RA8, A9, A6, A3, AP3, PsEND1, Modified 35S (inclusive of a conserved anther box sequence conferring tapetal specificity) and those specific to both pollen and anther tissues (NIN88, *bcp1*) (Table 2.2).

c) Mitochondrial target sequences: Approaches that aim at inducing male sterility by heterologous expression of CMS associated genes or exploitation of RNA editing, involve the need for efficient targeting of the candidate proteins into mitochondria, the site of action of these proteins. Normally, several proteins are encoded by the nuclear genome, synthesized on cytoplasmic ribosomes of a plant cell and translocated into distinct cellular compartments. These polypeptides are made as a precursor molecule with an amino-terminal extension termed leader peptide not found in the mature protein. These targeting sequences or the presequences or transit peptides, direct the proteins to the appropriate cellular compartments. Translocation of such proteins into chloroplasts or mitochondria requires a specific interaction between a receptor protein on the organelle surface and the leader peptide (Hartl *et al.*, 1989; Keegstra *et al.*, 1989; Sollner *et al.*, 1989). The general features of leader peptides important for such interaction appear to be highly conserved. But the targeting signal to the plant mitochondrial compartment requires more specific information within the signal sequence as plant cells contain chloroplasts in addition to the normal complement of eukaryotic cytoplasmic organelles. Protein targeting to plant mitochondria was initially worked out using the presequence of the gene for yeast mitochondrial tryptophanyl-tRNA-synthetase (MSW) in transgenic tobacco (Schmitz and Lonsdale, 1989).

Table 2.2 Examples of induction of transgenic male sterility using different approaches

Crop	Transgene	Transgene activity	Source of Transgene	Promoter used	Promoter specificity	Source of the promoter	Transit peptide with source	Approach for induction of transgenic male sterility	Reference
<i>Nicotiana tabacum</i>	RNase-T1	Ribonuclease	Chemically synthesized but homologous to gene from <i>Aspergillus niger</i>	TA29	Tapetum	<i>N. tabacum</i>	-	Expression of cytotoxic gene	Mariani <i>et al.</i> , 1990
<i>N. tabacum</i>	<i>barnase</i>	Ribonuclease	<i>Bacillus amyloliquefaciens</i>	TA29	Tapetum	<i>N. tabacum</i>	-	Expression of cytotoxic gene	Mariani <i>et al.</i> , 1990
<i>B. napus</i>	RNase-T1	Ribonuclease	Chemically synthesized but homologous to gene from <i>A. niger</i>	TA29	Tapetum	<i>N. tabacum</i>	-	Expression of cytotoxic gene	
<i>B. napus</i>	<i>barnase</i>	Ribonuclease	<i>B. amyloliquefaciens</i>	TA29	Tapetum	<i>N. tabacum</i>	-	Expression of cytotoxic gene	
<i>N. tabacum</i>	<i>barnase</i>	Ribonuclease	<i>B. amyloliquefaciens</i>	A6	Tapetum	<i>B. napus</i>	-	Expression of cytotoxic gene	Hird <i>et al.</i> , 1993
<i>N. tabacum</i>	<i>barnase</i>	Ribonuclease	<i>B. amyloliquefaciens</i>	PS1	Pollen	<i>Oryza sativa</i>	-	Expression of cytotoxic gene	Zhan <i>et al.</i> , 1996
<i>Brassica juncea</i>	<i>barnase</i>	Ribonuclease	<i>B. amyloliquefaciens</i>	TA29	Tapetum	<i>N. tabacum</i>	-	Expression of cytotoxic gene	Jagannath <i>et al.</i> , 2001
<i>N. tabacum</i>	<i>barnase</i>	Ribonuclease	<i>B. amyloliquefaciens</i>	PsEND1	Anther	<i>Pisum sativum</i>	-	Expression of cytotoxic gene	Roque <i>et al.</i> , 2007
<i>L. esculentum</i>	<i>barnase</i>	Ribonuclease	<i>B. amyloliquefaciens</i>	PsEND1	Anther	<i>P. sativum</i>	-	Expression of cytotoxic gene	
<i>Arabidopsis thaliana</i>	<i>barnase</i>	Ribonuclease	<i>B. amyloliquefaciens</i>	PsEND1	Anther	<i>P. sativum</i>	-	Expression of cytotoxic gene	
<i>B. napus</i>	<i>barnase</i>	Ribonuclease	<i>B. amyloliquefaciens</i>	PsEND1	Anther	<i>P. sativum</i>	-	Expression of cytotoxic gene	

Table 2.2 (cont.).

Crop	Transgene	Transgene activity	Source of Transgene	Promoter used	Promoter specificity	Source of the promoter	Transit peptide with source	Approach for induction of transgenic male sterility	Reference
<i>N. tabacum</i>	DsRIP	Ribosome inactivation protein	<i>Dianthus sinensis</i>	TA29	Tapetum	<i>N. tabacum</i>	-	Expression of cytotoxic gene	Cho <i>et al.</i> , 2001
<i>A. thaliana</i>	DTA	Potent inhibitor of translation	Diphthera toxin A	SLG	Stigmatic papillar cells and anther tapetum	Self Incompatibility locus of <i>Brassica</i>	-	Expression of cytotoxic gene	Thorness <i>et al.</i> , 1993
<i>Petunia hybrida</i>	A/S <i>chs</i>	Key enzyme of flavonoid biosynthesis	<i>P. hybrida</i>	Modified 35S (inclusive of a conserved anther box sequence)	Tapetum	CaMV (vector VIP 102)	-	Antisense inhibition	Van der Meer <i>et al.</i> , 1992
<i>A. thaliana</i>	A/S <i>bcp1</i>	Important for pollen development	<i>A. thaliana</i>	<i>Bcp1</i> promoter	Tapetum and pollen	<i>Brassica</i>	-	Antisense inhibition	Xu <i>et al.</i> , 1995
<i>A. thaliana</i>	A/S <i>bcp1</i>	Important for pollen development	<i>A. thaliana</i>	LAT52 promoter	Pollen	<i>L. esculentum</i>	-	Antisense inhibition	
<i>Solanum tuberosum</i>	A/S 55kDa subunit gene	NADH binding subunit of complex I	<i>S. tuberosum</i>	CaMV35S	Constitutive	CaMV	-	Antisense inhibition	Heiser <i>et al.</i> , 1997
<i>N. tabacum</i>	A/S PDH_E1 Alpha1	Mitochondrial pyruvate dehydrogenase essential for operation of TCA cycle	Sugarbeet	NA	Tapetum	NA	-	Antisense inhibition	Yui <i>et al.</i> , 2003

Table 2.2 (cont.).

Crop	Transgene	Transgene activity	Source of Transgene	Promoter used	Promoter specificity	Source of the promoter	Transit peptide with source	Approach for induction of transgenic male sterility	References
<i>Hordeum vulgare</i>	A/S SnRK1	Encodes SNF1-Related Protein Kinase-1 (regulation of carbon metabolism)	<i>Hordeum vulgare</i>	Glu-1D-1 promoter	Endosperm	<i>T. aestivum</i>	-	Antisense inhibition	Zhang <i>et al.</i> , 2001
Chinese cabbage	A/S CYP86MF	Encodes cytochrome P450	Chinese cabbage	CaMV35S	Constitutive	CaMV (pBI121 vector)	-	Antisense inhibition	Cao <i>et al.</i> , 2006
<i>Zea mays</i>	Inverted repeats of the Ms45 promoter	Expression in the anther tapetum during microspore development	<i>Z. mays</i>	Ubiquitin	Constitutive	<i>Z. mays</i>	-	Antisense inhibition - transcriptional gene silencing	Cigan <i>et al.</i> , 2005
Triticale	<i>susy</i>	Encodes sucrose synthase	NA	5' non-coding sequence of Osg6; Zmc13; β -gal gene	Anther and/or pollen specific	<i>O. sativa</i> , <i>Z. mays</i> , <i>T. monococcum</i>	-	RNA interference approach	http://www.unizh.ch/botinst/MoleculWebsite/bkeller_ho me.html
<i>N. tabacum</i>	<i>argE</i>	Converts N-acetyl-L-phosphinothricin to herbicide PPT	<i>E. coli</i>	TA29	Tapetum	<i>N. tabacum</i>	-	Conditional male sterility	Kriete <i>et al.</i> , 1996
<i>A. thaliana</i>	DTA ^{IS}	Encodes a mutant dipthera toxin active at 18°C but inactive at 25°C	Yeast	A9	Tapetum	<i>A. thaliana</i>	-	Conditional male sterility	Guerneau <i>et al.</i> , 2003

Table 2.2 (cont.).

Crop	Transgene	Transgene activity	Source of Transgene	Promoter used	Promoter specificity	Source of the promoter	Transit peptide with source	Approach for induction of transgenic male sterility	References
<i>N. tabacum</i>	<i>rolB</i>	Root loci b –increases auxin sensitivity in cells	<i>Agrobacterium</i>	DMC1	Anther and meiocytes	<i>Arabidopsis</i>	-	Alteration of hormonal balance	Cacchetti <i>et al.</i> , 2004
<i>N. tabacum</i>	modified PR- β -1,3-glucanase	Pathogenesis-related vacuolar β -1,3-glucanase - premature degradation of callose	<i>N. tabacum</i>	A3	Tapetum	<i>Arabidopsis</i>	N-terminal sequence of tobacco glucanase	Alteration of metabolic process	Worrall <i>et al.</i> , 1992
<i>N. tabacum</i>	modified PR- β -1,3-glucanase	Same as above	<i>N. tabacum</i>	A9	Tapetum	<i>Arabidopsis</i>	As above	Alteration of metabolic process	
<i>N. tabacum</i>	<i>Nin88</i>	Encodes for extracellular invertase involved in carbohydrate metabolism	<i>N. tabacum</i>	<i>Nin88</i> promoter	Anther and pollen	<i>N. tabacum</i>	-	Alteration of metabolic process by antisense expression	Goetz <i>et al.</i> , 2001
<i>N. tabacum</i>	<i>orf239</i>	Part of <i>pvs</i> mitochondrial CMS responsible for CMS	<i>Phaseolus vulgaris</i>	Lat52	Pollen	<i>L. esculentum</i>	<i>ap2-1</i> of <i>N. plumbaginifolia</i>	Heterologous expression of CMS associated gene	He <i>et al.</i> , 1996
<i>N. tabacum</i>	<i>orf239</i>	Part of <i>pvs</i> mitochondrial CMS responsible for CMS	<i>Phaseolus vulgaris</i>	sp	Constitutive	<i>Agrobacterium tumefaciens</i>	<i>ap2-1</i> of <i>N. plumbaginifolia</i>	Heterologous expression of CMS associated gene	
<i>O. sativa</i>	<i>orf79</i>	An abnormal <i>orf</i> possibly involved in CMS	BT cytoplasm of rice	CaMV 35S	Constitutive	CaMV (pCambia 1305.2)	Rf1b-5' from restorer of BT cytoplasm	Heterologous expression of CMS associated gene	Wang <i>et al.</i> , 2006

Table 2.2 (cont.).

Crop	Transgene	Transgene activity	Source of Transgene	Promoter used	Promoter specificity	Source of the promoter	Transit peptide with source	Approach for induction of transgenic male sterility	Reference
<i>A. thaliana</i>	<i>orf138</i>	Responsible for ogura CMS of <i>Brassica</i> and Radish	Synthetic	CaMV35S	Constitutive	CaMV (pBlue script)	With or without pre- β presequence of ATP-synthase of <i>N. plumbaginifolia</i>	Heterologous expression of CMS associated gene	Duroc <i>et al.</i> , 2006
<i>A. thaliana</i>	<i>orf138</i>	Responsible for ogura CMS of <i>Brassica</i> and Radish	Synthetic	A9	Tapetum	<i>A. thaliana</i>	With or without pre- β presequence of ATP-synthase of <i>N. plumbaginifolia</i>	Heterologous expression of CMS associated gene	
<i>N. tabacum</i>	<i>u-atp9</i>	Encodes unedited version of subunit 9 of ATP synthase	Wheat	35S	Constitutive	CaMV	<i>coxIV</i> from yeast	Exploitation of RNA editing	Hernould <i>et al.</i> , 1993
<i>A. thaliana</i>	<i>u-atp9</i>	Encodes unedited version of subunit 9 of ATP synthase	Wheat	ap3	Tapetum	<i>A. thaliana</i>	<i>coxIV</i> from yeast	Exploitation of RNA editing	Gomez-Casati <i>et al.</i> , 2002
<i>A. thaliana</i>	<i>u-atp9</i>	Encodes unedited version of subunit 9 of ATP synthase	Wheat	A9	Tapetum	<i>A. thaliana</i>	<i>coxIV</i> from yeast	Exploitation of RNA editing	
<i>O. sativa</i>	<i>u-nad9</i>	Encodes unedited version of subunit 9 of NADH oxidoreductase	NA	Ubiquitin	Constitutive	NA	At-mRBP1a from <i>A. thaliana</i>	Exploitation of RNA editing	http://www.avesthagen.com/docs/newsletters/ian_feb_2005.pdf

Abbreviations: *orf*, open reading frame; *nad*, subunit of NADH complex; *coxIV*, subunits of cytochrome oxidase; *atp9*, the subunit of the ATP-synthetase F_0/F_1 component, DTA^{ts}- Diphtheria toxin, DTA^{ts} - temperature sensitive diphtheria toxin

Later, many such transit peptides like *coxIV* (from yeast), *atp2-1* (from *Nicotiana plumbaginifolia*), *rfl* (from rice) have been used to induce transgenic male sterility by successfully targeting proteins into mitochondria (Table 2.2).

d) Transformation protocols: Availability of a reliable and efficient transformation system is a prerequisite in any transgenic development programme. This assumes paramount importance with respect to transgenic male sterility, as one has to ensure complete male sterility in the T₀ transgenic plants preferably carrying only one insert. This demands analyses of a large number of independent transformants to select the plants expressing the introduced gene in the required cell type at the effective level to ensure complete male sterility. Therefore, it is essential that an efficient protocol for transformation be developed in the crop of interest to increase the probability of obtaining the desired transgenic plant type. The different reports on safflower transformation have been reviewed in section 2.9.

e) Maintenance of the transgenic male sterile lines: Most of the transgenic male sterility systems, with the exception of recent chloroplast transformation method for induction of male sterility, are genetic (nuclear) in nature and thus they suffer from the inherent limitations of GMS system. To overcome this, mainly two strategies have been suggested and adopted. (i) Use of selectable markers such as herbicide resistance (linked to the male sterility inducing transgene) which could help in selecting the sterile plants during the maintenance of sterility: *Bar* gene conferring resistance to herbicide basta (phosphinothricin) has been used extensively for this purpose (ii) Use of an inducible sterility/fertility system: In the inducible male sterility system, the transgenic plant otherwise will be fertile and will become sterile only when induced with a chemical or a condition. This makes maintenance of sterility trait easy. In the inducible fertility approach, the normal

state is male sterile due to a block in the production of an essential metabolite (for fertility) and addition/application of that chemical metabolite to the male sterile plants restores fertility. Here, there is no need for 100% fertility as the amount of seeds set even with partial sterility will be sufficient enough to propagate the male sterile transgenic plants.

2.6 Different strategies for induction of transgenic male sterility

Due to the need for alternate systems of male sterility, transgenic approach is gaining importance in recent times. With molecular mechanisms underlying CMS being unraveled in recent times, a host of genes/proteins/metabolites present themselves as suitable candidates for induction of transgenic male sterility by different approaches. In all these attempts, disruption of the male gametophyte development or the release of the pollen grains has been the targets. The different approaches mainly adopted are reviewed below.

2.6.1 Expression of cytotoxic genes

Genetic ablation methodology in which a toxic gene is placed under the control of a tightly regulated promoter is a powerful tool for the analysis of developmental processes and the role of specific cell types in complex tissues. A very important aspect of this approach is the need for controlled expression of cytotoxic genes that can be achieved by use of cell-specific promoters that are active only in the target cell type. For inducing male sterility, the target cells should be those which will have profound effect on male gametophyte development like tapetum or other cells of anthers involved in either pollen formation or release.

The most popular as well as the first successful example of this approach (and also of genetically engineered male sterility) is the barnase-barstar system, where a ribonuclease gene from *Bacillus amyloliquifaciens* (*barnase*) was cloned under a tapetum specific promoter TA29. Introduction of this gene cassette into tobacco and rapeseed led to development of male sterile transgenics (Mariani *et al.*, 1990). The expression of *barnase* gene under TA29 promoter lead to selective ablation of the tapetal cell layer that surrounds the pollen sac which lead to disruption of normal pollen development. The barnase-barstar sterility-fertility restoration system has been adopted by several investigators with/without modification in several crops like *Brassica*, chicory, cauliflower, tomato, cotton, corn and wheat (Banga and Raman, 1998; Banga, 2006). Constructs with anther specific A6 promoter from *Arabidopsis* when fused to *barnase* gene and used for transformation lead to male sterile plants in tobacco and *Brassica napus* (Hird *et al.*, 1993). The promoter of a rice pollen-specific gene *PSI*, when fused to *barnase* gene and deployed in tobacco also lead to abnormal pollen morphology and male sterility in the transgenic plants (Zhan *et al.*, 1996). Male sterile lines were also generated in oilseed mustard (*Brassica juncea*) with a cytotoxic gene (*barnase*) in conjunction with either of two tapetum-specific promoters, TA29 and A9 (Jagannath *et al.*, 2001).

Apart from *barnase*, other cytotoxin encoding genes have also been tried for induction of male sterility (Table 2.2). Expression of the diphtheria toxin A (DTA), a potent inhibitor of translation, under the promoter of S locus Glycoprotein gene (derived from the self incompatibility locus of *Brassica*), affected papillar cell growth and anther development (Thorsness *et al.*, 1993). In another report, a ribosome inactivating protein (RIP) gene from *Dianthus sinensis* was used for induction of male sterility in tobacco. When TA29 promoter

from tobacco was fused to RIP cDNA and introduced into tobacco it led to the selective and complete destruction of tapetal tissue causing male sterility (Cho *et al.*, 2001). However, there are problems encountered with this system because of the toxicity of these genes. Even a slight and leaky expression of these genes in non-targeted cells could lead to unexpected problems.

2.6.2 Antisense inhibition of important genes

The antisense technology is based on blocking the information flow from DNA via RNA to protein by the introduction of an RNA strand complementary to (full or part of) the sequence of the target mRNA. The antisense RNA base pairs to its target RNA forming a double stranded RNA. This duplex may impair mRNA maturation and/or translation or alternatively may lead to rapid mRNA degradation. With the unraveling of RNA interference (RNAi), the mechanism through which double stranded RNA structure leads to gene silencing has become clearer. Male sterility could also be induced through impaired mitochondrial functioning using antisense technology. There are several reports on this aspect, some of which are reviewed here. In many of these cases, the researchers mainly aimed at understanding the role of these genes, but stumbled upon the fact that blocking them leads to male sterility.

Antisense inhibition of an essential enzyme leading to male sterility has been reported in transgenic petunia plants. In an attempt to inhibit the pigmentation in anthers of petunia, an antisense chalcone synthase (*chs*) gene under the control of a modified CaMV 35S promoter (modified by insertion of an anther box sequence) was expressed. Anthers from five out of 35 of these transgenic petunia plants were white due to inhibition of

pigment synthesis. Transgenic petunia plants with white anthers were male sterile due to an arrest in male gametophyte development (van der Meer *et al.*, 1992). This finding indicated that flavonoids play an essential role in male gametophyte development.

Another gene *bcp1* was found to be indispensable for pollen development, when antisense suppression of its expression (under *bcp1* promoter and LAT52 promoter) led to transgenic male sterile lines in *Arabidopsis* (Xu *et al.*, 1995) and *Brassica* (<http://regional.org.au/au/gcirc/4/493.htm>). Silencing of the gene (nuclear) for 55kDa NADH-binding subunit of complex I, by antisense repression in transgenic tobacco plants led to disturbed pollen maturation and thereby leading to reduced male fertility. This confirms the importance of complex I in mitochondrial energy production particularly in male generative tissues (Heiser *et al.*, 1997).

Yui *et al.* (2003) attempted to block anther development in sugar beet by antisense expression of the mitochondrial *PDH_E1A* gene in tapetal cells. This gene encodes pyruvate dehydrogenase (E1 component). The transgenic sugar beet plant carrying antisense *PDH_E1A* showed significantly reduced levels of the endogenous *PDH_E1A* mRNA and exhibited male sterility. Male sterility resulted from hypertrophy and vacuolation of tapetal cells, which invariably preceded the failure of pollen development. Transgenic barley expressing *SnRK1* gene (encoding Sucrose non-fermenting 1 related protein kinase, necessary for normal sucrose metabolism) in the antisense orientation under wheat Glu-1D-1 endosperm specific promoter, showed male sterile phenotype. It was found that the promoter also targeted anther cells where *SnRK1* was also expressed, leading to down regulation of the protein in anthers causing male sterility (Zhang *et al.*, 2001)

A high frequency of male sterile plants was obtained by constitutively (using maize ubiquitin promoter) expressing Inverted repeats (IR) of the Ms-45 promoter. Male sterile plants lacked MS-45 (reported to be important for male fertility) mRNA due to transcriptional inactivation of the target promoter (Cigan *et al.*, 2005). Attempts are in progress to develop transgenic male sterile triticale and wheat lines by down regulation of sucrose synthase (*susy*) gene under pollen and/anther specific promoters using hairpin RNA constructs(http://www.unizh.ch/botinst/Molec_Website/bkeller_home.html). The importance of several genes involved in the male gametophyte development being unraveled by recent studies implies that they could be knocked down by antisense approach to induce male sterility.

2.6.3 Inducing conditional male sterility

Another approach is to use an inducible system of cell ablation. This would allow normal development in permissive conditions but could be activated by a shift to non-permissive conditions. Some examples of this approach are discussed below.

A deacetylase enzyme of *E. coli*, N-acetyl-L-ornithine deacetylase encoded by the gene *arg E*, was identified to remove the acetyl group from N-acetyl-L-phosphinothricin (N-ac-ppt) giving a cytotoxic compound L-phosphinothricin (ppt, glyphosate). The *arg E* coding region was fused to a DNA fragment carrying sequences homologous to the tobacco TA29 promoter known to function exclusively in the tapetum and expressed in tobacco in a tissue specific manner (tapetal cells). When a non-toxic compound, N-ac-pt was sprayed on these transgenic plants, it was deacetylated to a cytotoxic compound, L-phosphinothricin in the tapetal cells leading to empty anthers (male sterility) and the female reproductive parts

remained unaffected and without N-ac-Pt treatment the plants turned out to be completely fertile making fertility restoration in the F_1 generation superfluous (Kriete *et al.*, 1996).

In another report, a gene encoding a temperature sensitive diptera toxin A chain polypeptide expressed under a *A. thaliana* tapetum specific A9 promoter, resulted in male sterile plants when grown at 18°C but were fully fertile at 26°C (Guerineau *et al.*, 2003).

2.6.4 Male sterility by inducing hormonal imbalances

Several studies on wild type and male sterile plants provide evidence of involvement of cytokinins and gibberellins in male reproductive organ development of flowering plants (reviewed by Sawhney and Shukla, 1994). Induction of male sterility by genetically manipulating endogenous hormone levels has been demonstrated in transformed tobacco plants carrying *rolc* (root loci c) gene of *Agrobacterium rhizogenes* under the control of CaMV35S promoter and flanked with a marker gene (Spena *et al.* 1987; Schmulling *et al.* 1988). In another experiment, Spena *et al.* (1992) used the tissue specific promoter 'tapI' and found that the inserted gene 'rolb' from *A. rhizogenes* (a gene which increases the auxin sensitivity of cells where it is expressed), greatly impaired the flower development in transgenic tobacco due to an increase in the levels of indole acetic acid and decreased levels of gibberellins in anthers. More recently, Cacchetti *et al.*, 2004 observed male sterility along with delayed anther dehiscence in transgenic tobacco plants having *rol b* gene expressed under *DMC1* (Deficient Meiotic Control 1) promoter of *Arabidopsis*. There is growing evidence that hormones play an important role in coordinating events within the flower leading to anther dehiscence (Sanders *et al.*, 2005). Hence it is logical to imagine that if the endogenous hormone levels are altered, male sterility could be induced. But, though the use

of hormonal alteration approach might provide valuable clues in elucidating the role of various hormones on particular tissue types or developmental stages, sufficient caution needs to be exercised as non-target cell types may exhibit the effect of hormones as a result of secondary response of expression of these transgenes.

2.6.5 Male sterility by alteration of metabolic processes

Attempts have been made to induce transgenic male sterility by down regulation or alteration of important metabolic process. Many examples covered in section 2.6.2 under antisense inhibition may also fall in this classification for induction of transgenic male sterility.

In CMS lines of *Petunia*, early appearance of active callose, a β -1,3- glucanase, in the anther locule leads to premature dissolution of the callose walls surrounding the microsporogenous cells resulting in male sterility. This aspect was mimicked in transgenic tobacco by engineering the secretion of a modified pathogenesis related vacuolar β -1,3- glucanase from the tapetum prior to the appearance of callose activity in the locule. Plants expressing modified glucanase under tapetum specific promoters (A3 and A9) exhibited partial to complete male sterility due to premature dissolution of the microsporocyte callose wall. The resultant microspores had an abnormally thin cell wall that lacked sculpturing. The tapetum showed hypertrophy. Male sterility could probably be due to bursting of the aberrant microspores at a time corresponding to microspore release (Worrall *et al.*, 1992).

The transportation of carbohydrates from photosynthetically active source tissues to developing sinks via apoplastic pathway is regulated by extracellular invertases. In tobacco, *Nin88* encodes for extracellular invertase, exhibiting specific temporal and spatial pattern of

expression. Tissue specific antisense repression of *Nin88* in transgenic tobacco caused male sterility by blocking pollen development during early stages of microsporogenesis (Goetz *et al.*, 2001).

2.6.6 Expressing male sterility causing genes from heterologous systems

Owing to the success of developing transgenic male sterile plants using different approaches, it was opined that if the gene responsible for CMS in one system is expressed in a heterologous system it might lead to male sterility. Hence, transgenic male sterile lines have also been developed by utilizing the information generated from investigations on molecular mechanisms of male sterility in naturally occurring CMS systems. Several studies have revealed that this maternally inherited trait is due to altered or aborted expression of one (or more) of the mitochondrial gene(s) or due to expression of new proteins which result from the formation of novel open reading frames (*orfs*) in the mitochondria of sterile lines (Horn *et al.*, 1991, Kohler *et al.*, 1991; Grelon *et al.*, 1994; L'Homme *et al.*, 1997; Kaul, 1998).

Recently, this knowledge has been used to obtain genetically engineered male sterile lines. As it is well established that these proteins are of mitochondrial origin and functional in the mitochondria, to induce male sterility the introduced protein must be functional in mitochondria. However, as protocols for genetic transformation of mitochondria are not available still, if any protein has to be made to function in mitochondria, the proteins produced in the cytosol will have to be targeted into mitochondria. It is known that several of the proteins functional in mitochondria are synthesized in the cytosol and then transported into mitochondria. Detailed studies of proteins imported into mitochondria from cytoplasm

have identified the peptide sequences at the amino terminal of the imported proteins, called pre-sequence or transit peptide, which guide the cytoplasmic protein into mitochondria. These sequences get cleaved once the proteins are docked into mitochondria. The pre-sequence of cytochrome c oxidase subunit IV from yeast has been studied extensively (Maarse *et al.*, 1984) and it has been used successfully to target the proteins into mitochondria in plants (Hernould *et al.*, 1993, 1998). During import of protein into the mitochondria, it has been demonstrated that the cytochrome c oxidase transit peptide is cleaved between Leu₂₅-Gln₂₆ amino acid residues. Therefore if a pre-sequence containing the first 26 residues of the transit peptide is present upstream of any protein, the fusion protein could be targeted into mitochondria. This has been already demonstrated in transgenic plants (Hernould *et al.*, 1993).

In common bean, the naturally occurring CMS has been ascribed to the 27 kDa protein encoded by the *orf239* sequence, which is expected to disrupt the pollen formation (Johns *et al.*, 1992; Abad *et al.*, 1995). When this *orf239* sequence was expressed as a nuclear transgene and the resultant ORF239 protein was targeted into mitochondria using an in-frame transit peptide (*atp2-1* of *N. plumbaginifolia*), it led to stable male sterile plants in tobacco demonstrating the utility of such sequences in obtaining male sterile lines in heterologous systems. It is surmised that crossing these plants with counterpart transgenic plants that contain antisense *orf239* as transgene could restore male fertility (He *et al.*, 1996).

In another experiment, in order to mimick the genetic effect of BoroII cytoplasm of rice, transgenic rice plants were developed using *orf79*, along with a mitochondrial transit signal from an *Rf* gene controlled by CaMV35S promoter. Though, T₀ plants showed semi

sterility phenotype (app. 50% of pollen grains being sterile), the T₁ generation plants clearly showed a 1:1 segregation ratio indicating gametophytic male sterility (Wang *et al.*, 2006).

Transgenic *Arabidopsis* plants expressing the mitochondrial *orf138* gene, responsible for ogura CMS of *Brassica* and radish (with or without the pre- β presequence of ATP-synthase of *N. plumbaginifolia*), under both constitutive (CaMV 35S) and tapetum specific (A9) promoters resulted in partial male sterile plants.

2.6.7 Male sterility by chloroplast engineering

In a recent breakthrough technology, induction of male sterility following the transformation of the tobacco plastid genome by a bacterial gene has been demonstrated (Khan, 2005; Chase, 2006). Transformation of the tobacco plastid genome with the *PhaA* gene from the bacterium *Acinetobacter* coding for β -ketothiolase under *psbA* promoter and other light regulated cis-elements (which will enhance translation of foreign genes under light) led to accelerated anther and pollen development relative to overall development of flower. This led to misshapen anthers and collapsed pollen. Ruiz and Daniell (2005) have proposed that fatty acid metabolism is particularly crucial to anther and pollen development, probably because it supports tapetal function. Expression of *phaA* in these tissues is thought to divert acetyl-CoA from synthesizing the fatty acid precursor malonyl CoA towards synthesizing PHB (Poly Hydroxy Butyrate) precursor aceto acetyl CoA resulting in failure of the tapetum and consequently failure of pollen development.

Among the various methods, the approaches which use DNA sequences derived from plant sources pose little concern with respect to biosafety compared to the expression of cytotoxic genes derived from different organisms. In most of these cases, the restoration

has been achieved by selectively blocking the expression of the gene causing male sterility (Mariani *et al.*, 1992; Kriete *et al.*, 1996; Schmulling *et al.*, 1993; Zabaleta *et al.*, 1996).

2.6.8 RNA editing for engineering male sterility

RNA editing is a post-transcriptional process that modifies the sequence of RNA through nucleotide insertion, deletion or modification. Hence, the amino acid sequence of the resultant protein becomes different from that encoded by the DNA. Insertion/deletion editing was first discovered in *Trypanosome* kinetoplasts, specialized mitochondria (Benne *et al.*, 1986) and has since been recognized in *Physarum* mitochondria and in several viruses. Few years later, RNA editing was discovered in plant mitochondria and it was demonstrated that C-to-U changes occurred in some positions when genomic and cDNA sequences were compared (Covello and Gray, 1989; Gualberto *et al.*, 1989; Hiesel *et al.*, 1989). These reports supported the hypothesis that mitochondria have some exceptions to the universal genetic code. It became clear that the so thought exceptions were due the process of RNA editing. For example, arginine codons (CGG) were some times detected in gene sequences at positions where tryptophan residues were highly conserved, and the possibility that CGG coded for tryptophan was suggested (Fox and Leaver, 1981). Analysis of cDNA sequences revealed that the unexpected CGG codons had been converted into UGG^{trp} codons by C-to-U editing.

2.6.8.1 Significance of RNA editing in plants

Conversion of C to U in majority of the cases leads to a change in the amino acid specified by a codon and the altered amino acid might be important for the structure and

function of the protein. For example, the three most common amino acid changes directed by editing in the *Arabidopsis* mitochondrial genome result in drastic changes of the hydrophobicity of the amino side chain: serine to leucine (93 changes); proline to leucine (80 sites); and serine to phenylalanine (47 sites) (Giege and Brennicke, 1999). Because these edited sites are mostly located at the first or the second nucleotide position of the codon, editing generally leads to evolutionarily conserved aminoacid residues. Thus, the primary function of RNA editing could be a genetic correction mechanism that corrects mutations in the genomic sequence so that the edited mRNA codes for the correct amino acid sequence that is required for the protein. These observations suggest that RNA editing is important to produce functional proteins. The importance of editing in the formation of functional proteins has also been hypothesized by several investigators (Gualberto *et al.*, 1989; Araya *et al.*, 1998).

A very conclusive example of the requirement of editing for protein function was shown by the requirement of RNA editing in the expression of *psbF*, a photosystemII polypeptide. The spinach *psbF* transcript is edited at a single C to cause a serine-to-phenylalanine conversion, but tobacco *psbF* mRNAs encode a phenylalanine residue and do not require editing at all at that site. The spinach editing site was introduced into the tobacco chloroplast *psbF* gene and the heterologous editing site could not be edited by the tobacco chloroplast editing machinery, thus creating a phenylalanine-to-serine mutation in the PsbF polypeptide. These transgenic plants exhibited a photo system II-deficient phenotype with retarded growth and pale green phenotype (De Block *et al.*, 1994). Thus failure to convert the editing site in the transgenic tobacco chloroplasts resulted in a mutant phenotype and this clearly emphasizes the genetic requirement for editing.

With the sequencing of several higher plant mitochondrial and chloroplast genomes, the extent of RNA editing in these organellar genomes has been elucidated. The *Arabidopsis* and rice mitochondrial genomes encode 441 and 491 C-to-U editing sites respectively (Giege and Brennicke, 1999; Notsu *et al.*, 2002). Higher plant chloroplast genomes sequenced so far have around 30 C-to-U editing sites (Kugita *et al.*, 2003; Maier *et al.*, 1995; Sugaira, 1995). The majority in these organelles are C-to-U editing sites whereas U-to-C editing sites are very rare. Exploitation of RNA editing to develop transgenic male sterile lines will be reviewed in detail as the present investigation utilized this feature for the development of transgenic male sterile lines.

2.6.8.2 Exploitation of RNA editing for engineering male sterility

Editing of mitochondrial genes is very crucial for functionality of corresponding proteins and this hypothesis was strengthened by several reports where transgenic male sterility has been induced by using unedited mitochondrial genes. Most of the mitochondrial genes encode components of multi-subunit complexes involved in electron transport or ATP synthesis reactions. Hence, the presence of unedited translation products of such genes within the organelle might lead to impairment in the assembly of these complexes which in turn may lead to mitochondrial dysfunction. This hypothesis has already been proven and exploited successfully to generate transgenic male sterile lines in sorghum and *Brassica* by using unedited mitochondrial genes and targeting their proteins into mitochondria (Howad and Kempken, 1997; Stahl *et al.*, 1994). Transgenic rice plants having upto 99.9% male sterility have been obtained using unedited *nad9* (*u-nad9*) sequence fused to the coding sequence of *Arabidopsis* mitochondrial transit peptide (At-mRBP1a) under the control of

ubiquitin promoter. Fertility of the transgenics was restored by crossing them to the transgenics expressing antisense for the *u-nad9* gene (http://www.avesthagen.com/docs/newsletters/jan_feb_2005.pdf). This technique is being tested for utility in sesame, mustard, sorghum, millet, tomato, okra and cotton. Though RNA editing has been reported for many mitochondrial genes, we have selected and used only three mitochondrial genes, viz., *atp6*, *nad3* and *atp9* in the present study and hence the information available regarding the editing in these three genes have been reviewed here.

The importance of RNA editing in the formation of functional protein in mitochondria has been harnessed for inducing male sterility in plants. RNA editing in *atp6* gene has been studied in plants like sorghum, rice, petunia etc. Several of these reports suggested that RNA editing of *atp6* transcripts is important for fertility. In sorghum, a transcript-specific loss of RNA editing was observed in anthers of CMS lines (Howad and Kempken 1997; Howad *et al.*, 1998). Loss of *atp6* RNA editing, as it occurs in sorghum CMS anthers, has been found to mimic mutations in human mitochondrial diseases. Mutations at specific positions in the *atp6* gene are the cause of neurogenic muscle weakness, ataxia and retinitis pigmentosa (NARP or Leigh syndrome) (Kempken *et al.*, 1998). The interaction of RNA processing and editing of B-*atp6* transcripts has also been found to play a role in controlling CMS expression and the restoration of fertility in rice (Iwabuchi *et al.*, 1993).

The *nad3* gene codes for subunit 3 of the mitochondrial NADH-ubiquinone-oxidoreductase complex. RNA editing in *nad3* has been studied in sunflower (Perrotta *et al.*, 1996), *Oenothera lamarckiana* (Schuster *et al.*, 1990), carrot (Rurek *et al.*, 2001), wheat (Gualberto *et al.*, 1991), sorghum (Howard and Kempken, 1997), *Petunia hybrida* (Lu and

Hanson, 1992) *etc.* The *nad3* gene, in most cases, is found to be co-transcribed along with *rps12* (encodes a protein of the mitochondrial small ribosomal subunit). The rice *nad3* is unique compared to other species (since the position of the start codon is conserved) as it is longer on the 5' side by 111 bp (Suzuki *et al.*, 1991).

The number of edited sites in *nad3* transcript of higher plants reported till date ranges from three sites in *Oryza sativa* (Suzuki *et al.*, 1991) to 21 sites in wheat (Gualberto *et al.*, 1991). Lu and Hanson (1992) have reported that the extent of editing of three edit sites of *nad3* varies among *Petunia hybrida* genotypes and a single nuclear gene is responsible for this variation. Gualberto *et al.*, 1991 have studied the presence of several partially edited *nad3-rps12* transcripts in wheat, but found that only the mature and completely edited transcripts are used for translation. This indicated that RNA editing is a post-transcriptional event. Rurek *et al.*, (2001) reported differences in editing of *nad3* transcripts from CMS and fertile carrots, though the differences were with respect to partial and silent edit sites. In radish, Rankin *et al.*, (2001) have observed that mitochondrial *nad3* might be a pseudogene and the corresponding functional gene might be in the nucleus.

The gene *atp9* codes for subunit 9 of the ATPase complex which is a membrane-associated protein subunit involved in the formation of proton-translocating F_0 portion of the ATPase in mitochondria. RNA editing has been studied in *atp9* of several plants. An interesting feature of *atp9* is that editing creates a stop codon that drastically reduces the polypeptide length to maintain a conserved carboxy terminal across species. This has been observed in *Oenothera* (Schuster and Brennicke, 1990), wheat (Begu *et al.*, 1990), petunia (Wintz and Hanson, 1991), sorghum (Salazar *et al.*, 1991), and tobacco (Hernould *et al.*, 1992). But in crops like soybean (Pesce and Grabau, 1993), the stop codon at the conserved

position is already genomically encoded. Begu *et al.*, 1990 have sequenced the ATP9 protein at the carboxyterminal region and confirmed that only the edited mRNA is translated into protein.

When unedited *atp9* (*u-atp9*) from wheat was expressed in tobacco and the resultant protein was targeted into mitochondria using the *coxIV* presequence from yeast, it resulted in male sterility (Hernould *et al.*, 1993) due to severe anther and pollen abnormalities in the transgenic plants (Hernould *et al.*, 1998)

Similar experiments have been carried out in *Arabidopsis* and male sterility was induced in transgenic plants expressing *u-atp9* (from wheat) gene under three promoters: constitutive 35S and the tissue specific promoters APETALA3 (*ap3*) and A9. Restoration of fertility of the transgenic male sterile lines was achieved by using antisense *u-atp9* transcripts. Also increased expression of some nuclear complex I genes was observed in the male sterile lines whereas their expression came down to normal levels in the restored *Arabidopsis* plants suggesting that upregulation of some nuclear complex I genes is triggered by mitochondrial dysfunction (Gomez-Casati *et al.*, 2002).

2.7 Restoration of fertility in transgenic male sterile lines

Male sterility system could be exploited only if there is a good fertility restoration mechanism/strategy so that the hybrid plants would be male fertile and they can self fertilize. This is absolutely essential if seed is the economical part of the plant and the crop is predominantly a self fertilized one. This is of greater significance especially because transgenic male sterility is a dominant mutation and plants expressing the transgene continue to be male sterile. Fertility restoration could be achieved effectively if the functionality of

protein encoded by the transgene is abolished. Though several investigators have reported the development of transgenic male sterile plants (Table 2.2), very few have reported the development of appropriate restorer lines which are essential for applicability of the system for hybrid seed production programmes. Also, with few exceptions, most of these studies are in the model plants tobacco and *Arabidopsis*. Though the importance of these investigations as proof of concept in model plants cannot be downplayed, it is much essential to extrapolate these approaches in other crop plants where hybrid vigour needs to be exploited. Hence development of a functional and viable male sterility-restoration system is very important. Also, in cases where fertility restoration has been reported, the problems in achieving complete restoration has been highlighted with very low frequency of restorer lines obtained. Restoration could be effected at protein level, RNA level and DNA level.

2.7.1 Restoration effected at the protein level

The most successful of all systems so far, has been the *barnase-barstar* system, which has been applied to a wide number of crops. As discussed earlier in this system, male sterility is induced due to the expression of a cytotoxic ribonuclease gene called *barnase*. Therefore it was hypothesized that the cytotoxic effect of *barnase* gene could be eliminated if the specific/corresponding ribonuclease inhibitor that could stoichiometrically bind to the ribonuclease and inhibit its activity, is also expressed in the same plant. With this logic, transgenic plants for fertility restoration were developed with *barstar*, a ribonuclease inhibitor gene, from *Bacillus amyloliquifaciens* under TA29 promoter.

Transgenic oilseed rape (*B. napus*) plants expressing the *barstar* gene under TA29 promoter, when crossed with the TA29-*barnase* male sterile plants, restored fertility in the

progeny. Analysis of the F₁ and F₂ progeny from these crosses indicated that TA29-*barstar* gene functions as a dominant suppressor and is inherited stably (Mariani *et al.*, 1992). Similarly, transgenic male sterility system has been developed in *Brassica juncea* (Jagannath *et al.*, 2001, 2002). Three agronomically superior male sterile lines among these transgenic *B. juncea* plants were selected and a search for suitable restorer transgenic lines, culminated in identification of a complete functional male sterility/restorer system in *B. juncea*. But most of the crosses between *barnase* and *barstar* expressing lines were still male sterile and weak expression of the transgene in vegetative tissues led to yield reduction (Jagannath *et al.*, 2002) with only one of the 54 crosses giving complete restoration. Inability to identify more male sterile – restorer combinations has been opined to be due to insufficient levels of *barstar* protein in the restorer lines at the appropriate developmental stage which failed to successfully inhibit *barnase* activity in the F₁ plants. This necessitated development of alternate strategies for enhancing and extending temporal expression of restorer gene (*barstar*) so that more number of effective restorer lines could be developed. It was found that presence of two independent transcription units for the *barstar* gene (with A9 and TA29 promoters) gave higher frequencies of restoration compared to presence of single transcription unit or use of a chimaeric tissue specific promoter (combination of regulatory elements of A9 and TA29). This was due to extended temporal expression of the *barstar* protein since these two promoters have overlapping expression profiles (Bisht *et al.*, 2004). In an alternate strategy, Burgess *et al.*, 2002 used a two component system of *barnase* to develop male sterile tomato lines (discussed earlier) in which the fertility could be restored by crossing the male sterile lines with any normal (untransformed) pollen parent.

The limitation of restoration at protein level is the availability of cognate inhibitory proteins, antibodies that completely stop the functioning of sterility inducing protein(s). It can be envisaged that identification/development of such a system is a daunting task given the complexity of identifying antibody encoding genes that usually result from complicated 'gene shuffling' mechanisms.

2.7.2 Restoration effected at RNA level

As per the 'Central Dogma' of molecular biology, genes are active only when they transcribe and the transcript leads to protein production through translation process. Therefore, if the target transcripts are eliminated or if the translation process is inhibited, then the specific encoded protein will not be produced. It is essential to eliminate the target messenger RNA completely to ensure 100 per cent silencing of the transgene and thereby achieve restoration.

In earlier studies, for gene silencing, antisense technology has been utilized (Gray *et al.*, 1992; Knutzen *et al.*, 1992; Kinney 1996). This (antisense) technology has also been utilized for restoring fertility in transgenic male sterile plants. Male sterile plants developed by expression of *u-atp9* when crossed with transgenic plants expressing antisense version of this gene (*as-atp9*), could effectively restore fertility in tobacco (Zabaleta *et al.*, 1996) as well as in *Arabidopsis* (Busi *et al.*, 2006). Analysis of F₁ and F₂ progeny of tobacco plants indicated that the *as-atp9* gene blocks the expression of the *u-atp9* by functioning as a dominant restorer gene (Zabaleta *et al.*, 1996).

In another system, male sterile tobacco plants were generated by prematurely expressing a modified form of PR-1,3 glucanase in tapetal cells of the anther under the

control of A3 and A9 tapetum specific promoters (Worrall *et al.*, 1992). Varying levels of fertility restoration was obtained using sense and antisense PR-glucanase constructs under the control of A9 promoter by retransformation of A9-PR-glucanase male sterile plants (Hird *et al.*, 2000). This partial restoration could be due to lower efficiency of antisense RNA technology in completely suppressing the sterility-inducing gene.

The relatively low frequency of PTGS achieved with anti-sense and co-suppression requires development of a large number of independent transgenic events in order to select appropriate transgenic lines exhibiting sufficient degree of target gene suppression and identify the best transgenic lines that is agronomically equivalent to parent line chosen for transformation. This can present a major limitation, particularly in species that have low transformation and regeneration frequency.

Plant biologists have developed efficient gene silencing strategies such as ihp-RNA (intron interrupted hairpin RNA) and SHUTR (Silencing by heterologous 3' untranslated Region) (Smith *et al.*, 1990; Brummell *et al.*, 2003) which could be applied to genetically engineered male sterility-fertility restoration systems. Extensive reviews regarding these strategies are available (Mansoor *et al.*, 2006; Bonnet *et al.*, 2006; Baulcombe, 2005; Waterhouse and Helliwel 2002).

2.7.3 Restoration effected at DNA level

Another efficient method of fertility restoration could be elimination of the male sterility inducing gene cassette from the genome (at the DNA level). This approach is especially relevant to male sterile plants developed through antisense approach or by expression of genes encoding cytotoxic proteins (like the diphtheria toxin) for which

cognate inhibitor proteins (which could have been used for restoration) have not been identified. Since gene-knockout system through homologous recombination is not available in plants, a similar strategy has been developed by exploiting the site specific recombination systems such as FLP-FRT and CRE-LOX taken from 2 μ m plasmid of yeast and bacteriophage respectively. Combining any of the transgenic male sterility induction approaches with a site-specific recombination system in order to eliminate the sterility inducing gene leads to an effective restoration of fertility.

Site specific recombinases are enzymes that recognize specific DNA sequences and in the presence of two such recombination sites, they can catalyze the recombination of DNA strands (Ow and Medberry, 1995). The feasibility of one such system, FLP-FRT from the 2 μ m plasmid of yeast, for developing *Arabidopsis* hybrids, has been reported by Luo *et al.*, 2000. Male sterile plants induced by using the antisense of the *bcp1* gene flanked by FRT sites, when crossed with pollen of FLP containing plants, produced viable seeds that gave rise to normal progeny. The antisense expression cassette of *bcp1* had been excised in these hybrid plants.

Another example is the *Cre/loxP* system demonstrated in tobacco (Bayer and Hess, 2005). A stilbene synthase (*Sts*) transgene flanked by *loxP* recognition sites for Cre-recombinase was used to develop male sterile plants. Crossing these plants with tobacco lines expressing the *cre* recombinase transgene resulted in site-specific recombination in the hybrids leading to restoration of fertility. Both these approaches reported 100% recombination efficacy when the parent contained a single copy transgene per haploid genome. Hence this system appears to be promising in hybrid breeding programs with agronomically important crop plants. Thus as mentioned earlier, this method of restoration

becomes, an essential and viable strategy to restore fertility where sterility is induced using antisense approach to suppress some essential gene(s) implicated in normal male gametophyte development.

2.8 Tissue culture of safflower

In most plant transformation methods, a whole plantlet is regenerated from isolated plant cells or tissues which have been genetically transformed. Hence, environment and growth medium have to be manipulated during *in vitro* culture of explants not only to ensure a high frequency of regeneration but also to get regenerable cells that are readily accessible to gene transfer.

It is known that growth hormones play an important role during *in vitro* culture of plants. The balance in the level of auxin and cytokinin determine the morphogenetic pattern. A relatively high auxin to cytokinin ratio stimulates rooting, whereas low auxin to cytokinin ratio produces shoots and almost equal proportion of each results in undifferentiated callus (Skoog and Miller, 1957). Successful regeneration of a plant *in vitro* is governed by several factors like nutrient medium, growth hormones, physical environment *etc.* Studies have been taken up in tissue culture of safflower, but with limited success especially for rooting and acclimatization. Here we review the tissue culture work done so far in safflower.

2.8.1 Shoot induction in safflower

Both direct as well as callus mediated shoot regeneration via the organogenetic and embryogenetic pathways have been reported for several safflower genotypes. Murashige and Skoog salt medium (Murashige and Skoog, 1962) has proved to be the best among all the

basal media tried for morphogenetic response (Prasad *et al.*, 1991; Chatterji and Singh, 1993).

Among the hormonal combinations tried, the best response in terms of frequency of responding explants has been with TDZ and NAA combination with tested genotypes and all tissue types (Orlikowska and Dyer, 1993; Radhika *et al.*, 2006). BA singly or in combination with NAA has also given good shoot regeneration via both organogenesis and embryogenesis (George and Rao, 1982; Tejavathi and Anwar, 1984; Chatterji and Singh, 1993; Rani *et al.*, 1996; Mandal *et al.*, 1995). Other cytokinins such as kinetin, 2-isopentyl adenine and zeatin singly and in combination have been tried but did not show much response (George and Rao, 1982; Radhika *et al.*, 2006).

2.8.2 Root induction in safflower shoots

Root induction in regenerated shoots has been a major bottleneck in safflower tissue culture. Several workers have suggested improvements to enhance the frequency of rooting like high sucrose levels upto 9% in the rooting medium (Tejavathi and Anwar, 1987; Nikam and Shitole, 1999), high sucrose (9%) along with 1 mg/l each of NAA and riboflavin (Orlikowska and Dyer, 1993), incorporation of 2,4,5, trichlorophenoxypropionic acid, (Tejavathi and Anwar, 1993; Baker and Dyer, 1996) and root induction with *Agrobacterium rhizogenes* (Baker and Dyer, 1996). A seven-day exposure of shoots to 10 mg/l IBA in root induction media, followed by incubation in media with 15 g/l sucrose and 1 g/l activated charcoal for 21 days enhanced rooting frequency and reduced shoot hyperhydricity (Baker and Dyer, 1996). Use of IBA and phloroglucinol in combination as well as low NAA (0.5 mg/l), gave a high frequency of rooting in safflower shoots (Rao *et al.*, 2007).

2.8.3 *In vitro* induction of capitula

Another interesting feature of safflower tissue culture is the *in vitro* induction of capitula on media with appropriate combination of growth regulators (Tejovathi and Anwar, 1984; Radhika *et al.*, 2006). Capitula were induced frequently on media supplemented with BA + NAA and at a low frequency on media fortified with kinetin (Tejovathi and Anwar, 1984). *In vitro* produced flowers (in BA + NAA combination) were normal with normal pollen production and seed set.

Protocol for induction of somatic embryo in safflower has been reported (Mandal *et al.*, 1995; Mandal and Guptha, 2002). There is an immediate need for optimization of parameters for enhancing embryo to plantlet conversion frequency, somatic embryo maturation and encapsulation not only for use in large scale plant propagation but also in genetic transformation due to low probability of recovery of chimaeric tissues.

2.9 Genetic transformation in safflower

Owing to the development of different methods of plant transformation during the last two decades, transgenics are being widely used in both basic and applied studies in plant biology. But very few reports on safflower transformation are available in literature. The methods of transformation include callus-mediated regeneration from transformed seedling tissues (Ying *et al.*, 1992; Orlikowska *et al.*, 1995; Rao and Rohini, 1999) or whole plant development from transformed embryos through a method termed *in planta* approach (Rohini and Rao, 2000).

Rohini and Rao (2000) reported transformation of embryo axes of germinating seeds by pricking with sterile needle and then co-cultivation with *Agrobacterium* culture for 24

hours. Subsequent decontamination with cefatoxime for 1 hour and then transfer of the germinating seedlings to moistened soilrite ensured normal healthy plants which were later transferred to pots in green house. The transformation frequencies were 5.3% and 1.3% for 'A-1' and 'A-300' genotypes respectively.

Explants of safflower cultivar 'Centennial' inoculated with *Agrobacterium tumefaciens* containing *nptII* and *gus* genes produced kanamycin-resistant calli from which buds were regenerated. Transformation and stable integration was confirmed by GUS assay and DNA hybridization in kanamycin-resistant calli, and GUS assay in regenerated shoots (Ying *et al.*, 1992). The effects of co-cultivation conditions on transformation efficiency and direct shoot regeneration from seedling explants of safflower cv. 'centennial' have been reported (Orlikowska *et al.*, 1995).

Presence of the introduced gene in these experiments was initially confirmed through GUS histochemical assay. Molecular confirmation was done through PCR analyses and /or Southern analyses (Ying *et al.*, 1992; Orlikowska *et al.*, 1995). Techniques like non-denaturing PAGE (for neomycin phosphotransferase activity), western blotting and dot blot hybridization (Rao and Rohini, 1999) were also employed.

2.10 Cloning and transformation initiatives in safflower

Leaf spot of safflower incited by *Alternaria carthami* causes significant yield losses worldwide in safflower. Brefeldin A (BFA) which has a pivotal role in *A. carthami* infection of safflower, could be detoxified by esterase from *Bacillus subtilis*. Hence this gene was cloned, sequenced and characterized for use in *Agrobacterium*-mediated transformation of safflower (Matern and Kneusel 1993), though this attempt was not successful. Effectiveness

of TA29 promoter (for expression in tapetal tissues) and *coxIV* pre-sequence (for mitochondrial targeting) has been studied by cloning these genes along with *gus* gene in appropriate constructs and developing safflower transgenics with them (Kalyani *et al.*., 2007).

SemBioSys Genetics Incorporation (www.sembiosys.ca), a Canadian plant biotechnology company is involved in use of safflower for production of various pharmaceutical, cosmaceutical and nutraceutical products like human insulin, Apolipoprotein A1, growth hormones, high value fatty acids (such as GLA, DHA) antibodies etc. Appropriate genes towards this aim, have been cloned and are being used for safflower transformation.

2.11 Analysis of transgenic plants

Detailed analysis of transgenic plants is a very crucial component of genetic engineering. Transgenic plants (even when same construct is used and same conditions are provided) show very high degree of variation that could arise at three levels i.e., tissue culture, transformation and breeding. Almost all transformation procedures involve tissue culture to regenerate complete plantlets from transformed cells. Prolonged exposure of explants to hormones is known to induce several genetic and epigenetic changes (somaclonal variations). Transformation is another major contributor to variations arising in transgenic plants. Independent transgenes carrying the same cassette of foreign DNA sequence will behave differently depending on their genomic context in the host genome (position effect). Another complication arises due to differences in copy number of transgenes present in independent transgenic plants. Individual insertions could also carry

multiple copies of the transgene. Selfing or backcrossing of the primary transgenics (T_0) leads to variations in the progeny depending on the segregation pattern of the transgene(s). All these variations in transgenics make their analysis a complicated issue. However, use of appropriate controls and proper molecular and genetic analysis can be very effective in selecting single insertion transgenics as well as apportioning the transgene effect from all other effects (Bhat and Srinivasan, 2002).

2.11.1 Analysis of safflower transgenic plants

There are very few reports on development of transgenic plants in safflower. In all of these reports PCR assays have been used to confirm the transgenic status of plants and southern hybridizations further indicate the number of inserts. Histochemical assays for localization of GUS expression have also been done (Ying *et al.*, 1992; Orlikowska *et al.*, 1995). Rohini and Rao, 2000 analysed the T_0 and T_1 plants by southern hybridization and confirmed the inheritance and integration of the transgenes. However the Mendelian inheritance could not be deduced due to the small number of seeds obtained.

2.11.2 Analysis of transgenic male sterile and restorer plants

2.11.2.1 Phenotypic analysis

All the studies involving transgenic male sterile lines analyze plants primarily for the floral morphology and seed set. Generally, shape and color of flowers including corolla, anthers and pistil are carefully observed. Also some vegetative traits such as plant height, number of nodes, number of leaves etc., are observed to identify any abnormality that might be exhibited due to the transgene expression. In tobacco, the transgenic male sterile plants

demonstrate a compacted floral head and collapsed grayish anthers that appear receded below the level of stigma compared to normal fertile flowers (He *et al.*, 1996; Hernould *et al.*, 1993; Hernould *et al.*, 1998).

2.11.2.2 Cytological analysis

Transgenic plants male sterile and /or restorer are analyzed for pollen fertility or sterility (by pollen staining) or pollen viability (by pollen germination). Pollen staining is done by using aniline blue (He *et al.*, 1996) or fluorescein diacetate (Harrison and Harrison, 1970). Pollen viability is evaluated by the germination capacity of the pollen in a pollen germination medium usually containing sucrose and boric acid (Hernould *et al.*, 1993).

Studies on microsporogenesis in the anthers (of various developmental stages) of transgenic plants by using light microscopy and transmission electron microscopy could reveal the exact stage and type of abnormalities in the tapetal cells of the transgenic male sterile plants (He *et al.*, 1996; Hernould *et al.*, 1998). Hernould *et al.*, (1998) have identified prophase I of meiosis as the stage where degeneration begins, culminating in very meiocytes being formed in anthers of transgenic male sterile plants compared to control tobacco plants. They observed irregular tapetal cell layer with aberrant morphology, high degree of vacuolization and also condensed as well as deformed nuclei in the transgenic plants.

2.11.2.3 Molecular analysis

PCR analysis is generally done in all plants of T₀, T₁ and further generations. Appropriate primers combinations are chosen so as to confirm all the components (including the gene for selection of transformants) of the construct and their proper orientation. Also

care should be taken that only the transgene is amplified and not an endogenous gene (in cases where transgene has complete or partial homology to an endogenous gene). This can be done by selecting primer combinations in such a way that the endogenous gene is excluded but not the transgene (Hernould *et al.*, 1993).

To prove the integration of the foreign gene into the host genome usually Southern blotting (or DNA blotting) is done. Apart from knowing the number of inserts so that single insertion transgenics can be identified and used for further studies, Southern blotting can also be effectively used to detect truncations, rearrangement or multicopy insertions at some loci (Bhat and Srinivasan, 2002). Several studies involving analysis of transgenic male sterile plants have used PCR and southern blotting (Hernould *et al.*, 1993; He *et al.*, 1996; Zabaleta *et al.*, 1996).

Northern blotting and/or RT-PCR analyses are used to assess the steady state levels of transgene mRNA present in the tissues which indicates the degree of transgene expression. This also gives an indication of the spatial and temporal effectiveness of the promoter utilized. For example, results of RT-PCR indicated a higher expression of *u-atp9* genes in flowers of the transgenic *Arabidopsis* plants when A9 and *ap3* promoters were used but very low expression was detected when constitutive 35S promoter was used. During such studies, the expression of a housekeeping gene, in this case *elf4A*, indicates that the tissues are healthy and not deteriorated (Gomez-Casati *et al.*, 2002). Several studies involving transgenic male sterile and/or restorer lines have utilized these techniques to analyze the transgene expression (Hernould *et al.*, 1993; He *et al.*, 1996; Zabaleta *et al.*, 1996; Busi *et al.*, 2006; Gomez-Casati *et al.*, 2002). The transgenic plants showing high expression levels could be utilized for further studies. *In situ* mRNA hybridization has also

ANGRAU Central Library
HYDERABAD-500 030.
ACC. No. D-8088...
Date.....9.1.88.....

been used to investigate the transgene expression specifically in the male reproductive organs (Hernould *et al.*, 1998).

Immunoblotting (or western blotting) has been used to analyze the production of transgenic protein in appropriate cellular fractions. Substantial differences in polypeptide lengths as well as amount of proteins can also be detected by this method (Heiser *et al.*, 1997; Hernould *et al.*, 1993). Presence of the protein expressed by the transgene has also been detected effectively by immunolocalization. Use of appropriate probes can confirm all the component proteins (like transit peptide) and also differentiate the transgenic protein from any endogenous protein (Hernould *et al.*, 1993; Hernould *et al.*, 1998). Enzyme-linked immunosorbent assays (ELISA) has also been used to detect the expected proteins in transgenic plants (He *et al.*, 1996). Apart from the above studies, the effect of the transgene expression on respiratory function could also be analyzed by determining the oxygen consumption rate in the transgenic plants (Hernould *et al.*, 1998). Balk and Leaver (2001) have used standard electrode assays, cytochrome c activity and respiratory control ratio (RCR) to determine the respiratory properties of isolated mitochondria.

2.11.2.4 Genetic analysis

Handling of primary transgenics must be done in a thoughtful manner so as to analyze the transgenics effectively for the desirable phenotype. The general practice is to select the progenies (T_1 plants) obtained after self fertilization of primary transgenics (T_0 plants) for analysis. The resultant T_1 progenies are a complex mix of genotypes including homozygous, homozygous and non-transgenic plants as well as transgenics with different dose of transgene (arising by segregation on multiple insertion transgenics in T_0) that makes

analysis a cumbersome process. An effective strategy to overcome and simplify genetic analysis is to effect reciprocal backcross between T_0 plants and untransformed plants of the parent varieties. Back cross progenies (BC_1F_1) of multi locus insertion transgenics will give rise to single locus transgenics if the insertions are not tightly linked. The proportion of both single insertion transgenics and non-transgenics will be higher in BC_1F_1 (1:1 ratio) than in T_1 progenies. All the transgenic plants in the BC_1F_1 generation will be in hemizygous state, hence copy number, position effects can be easily discerned. Since single unaltered transgene insertions are ideal for analysis, it is necessary to check the presence of multi copy random insertions and other rearrangements at a given locus (Bhat and Srinivasan, 2002).

Transgenic male sterile plants obtained in T_0 generation (since they cannot be selfed) are crossed with untransformed plants to obtain the BC_1F_1 where they show a 1:1 segregation for transgenics and non-transgenics. The fertile transgenics (that have the transgene but still show fertility) as well as transgenics developed for restoration of fertility are selfed and they show 3:1 segregation ratio. Single insertion and homozygous male sterile and restorer plants are selected and crossed to effect 100% fertility restoration (Zabaleta *et al.*, 1996).

CHAPTER III

MATERIALS AND METHODS

CHAPTER III

MATERIALS AND METHODS

The present investigation was carried out at the Biotechnology Laboratory, Directorate of Oilseeds Research, Rajendranagar, Hyderabad with an objective of developing transgenic male sterile and fertility restorer lines in safflower. The strategy involved identification of mitochondrial genes which undergo substantial editing in safflower, development of suitable constructs for induction of male sterility as well as fertility restoration using unedited versions of the identified gene(s), genetic transformation of safflower as well as tobacco with these constructs and confirmation of the transgenics. It was intended that nuclear expression of unedited mitochondrial gene(s) (which code for important mitochondrial complexes) and targeting of the resultant proteins into mitochondria using a transit peptide would lead to impairment of mitochondrial functioning and thereby lead to male sterility. This strategy has been adopted to investigate the induction of transgenic male sterility in safflower and tobacco.

In the present investigation, three mitochondrial genes viz., *atp6*, *nad3* and *atp9* were studied for their RNA editing status in safflower. Of these, latter two gene transcripts revealed substantial editing and hence, were used to develop the gene constructs. Constructs for induction of male sterility were developed by cloning the unedited (genomic) versions of these genes under TA29 promoter (for specific expression in tapetal tissues of anther) and *nos* terminator along with *coxIV* presequence (which would help in targeting the protein into mitochondria). Towards development of fertility restorer lines, antisense and RNAi constructs using the above genes were developed. A total of ten constructs were developed

of which four were used for genetic transformation of safflower and tobacco. Transgenic plants were obtained for all the constructs in safflower and tobacco. These plants were confirmed by PCR analyses, leaf assays of phosphinothricin/hygromycin sensitivity, morphological observations and pollen studies. The materials used and the methods followed during the present investigation are outlined here.

3.1 Materials

3.1.1 Plant Material

Safflower (*Carthamus tinctorius* L.) cultivar HUS305 from the Directorate of Oilseeds Research (DOR), Hyderabad and Tobacco (*Nicotiana tabacum* L.) cultivar Xanthi from International Crop Research Institute for Semi-arid Tropics (ICRISAT), Hyderabad were used in the present study.

3.1.2 Equipment

The equipments used in the study are listed in Table 3.1.

3.1.3 Media

For plant tissue culture:

Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) was used as the basal medium for tissue culture and transformation of safflower as well as tobacco. For preparation of medium, either the readymade media devoid of hormones (Himedia, India) or stock solutions of component chemicals were made and used for making required working medium (by mixing them in required proportions in distilled water to get the desired

Table 3.1 Equipments used in the present investigation

Equipment	Company or Supplier
Centrifuges	Heraeus, Sorvall
Ligation bath	Genei, Bangalore
Electrophoresis units	Biorad, Consort
Thermal cycler	Master Cycler (eppendorf)
Gel documentation system	Syngene
Electroporator	BioRad
Vacuum/pressure pump	Millipore
Water bath	Haake
Laminar air flows	Klenzaid's air flow systems
Incubator-shaker	Julabo
Autoclaves	Sanyo, LabTech
Hot air oven	Mas-tek

concentrations). The composition of MS medium is given in Table 3.2. The basal MS medium with full strength or half strength of the components with suitable hormones, antibiotics/selection agents were used during different stages of transformation and regeneration.

For culture and maintenance of bacterial clones and strains:

Luria Bertani (LB) liquid medium and solidified medium (LA) were prepared from the products supplied by Himedia Company and used for culture of *E. coli* strains. Terrific Broth (TB) containing bacto-tryptone 1.2%, bacto-yeast extract 2.4%, glycerol 0.4%, potassium dihydrogen orthophosphate 0.231%, potassium hydrogen phosphate dibasic 1.25%, TA (TB medium supplemented with 0.8-1.5% agar) media as well as YEP [0.5% yeast extract, 0.5% bacteriological peptone, 0.3% $(\text{NH}_4)_2\text{SO}_4$, 0.3% KH_2PO_4 , 2% glucose] were used for culturing as well as maintaining *Agrobacterium* strains. Keto-Lactose medium (Lactose 1%, yeast extract 0.1% and bacto agar 2%) was also used to reconfirm the *Agrobacterium* clones.

3.1.4 Hormones

The details of the hormones used in the present investigation are given in Table 3.3.

3.1.5 Antibiotics / Herbicide

The details of the antibiotics and the herbicide phosphinothricin used in the present investigation are given in Table 3.4.

Table 3.2 Composition of MS medium

Stock	Constituents	Actual conc. (mg l ⁻¹) × magnificant	Vol. of stock solution (ml)	Vol. of stock solution used (ml/l)
A	Macronutrients			
	NH ₄ NO ₃	1650 × 10	500	50
	KNO ₃	1900 × 10		
	MgSO ₄ .7H ₂ O	370 × 10		
	KH ₂ PO ₄	170 × 10		
B	Calcium Chloride		100	10
	CaCl ₂ .2H ₂ O	440 × 10		
C	Micro-nutrients			
	KI	0.83 × 50	100	2
	H ₃ BO ₃	6.20 × 50		
	MnSO ₄ .4H ₂ O	22.3 × 50		
	ZnSO ₄ .7H ₂ O	8.6 × 50		
	Na ₂ MoO ₄ .2H ₂ O	0.25 × 50		
	CuSO ₄ .5H ₂ O	0.025 × 50		
	CoCl ₂ .6H ₂ O	0.025 × 50		
D	Iron-EDTA		100	10
	Na ₂ EDTA.2H ₂ O	37.3 × 10		
	FeSO ₄ .7H ₂ O	27.8 × 10		
E	Vitamins			
	Glycine	2.0 × 100	100	1
	Nicotinic Acid	0.5 × 100		
	Pyridoxine-HCl	0.5 × 100		
	Thiamine-HCl	0.1 × 100		
F	Myo-Inositol			
	Myo-Inositol	100 × 10	100	10
	Sucrose			
	Agar	30,000	Added directly	Added directly
	pH	7,000 5.8		

Table 3.2 Composition of MS medium

Stock	Constituents	Actual conc. (mg l ⁻¹) × magnificant	Vol. of stock solution (ml)	Vol. of stock solution used (ml/l)
A	Macronutrients			
	NH ₄ NO ₃	1650 × 10	500	50
	KNO ₃	1900 × 10		
	MgSO ₄ ·7H ₂ O	370 × 10		
	KH ₂ PO ₄	170 × 10		
B	Calcium Chloride		100	10
	CaCl ₂ ·2H ₂ O	440 × 10		
C	Micro-nutrients			
	KI	0.83 × 50	100	2
	H ₃ BO ₃	6.20 × 50		
	MnSO ₄ ·4H ₂ O	22.3 × 50		
	ZnSO ₄ ·7H ₂ O	8.6 × 50		
	Na ₂ MoO ₄ ·2H ₂ O	0.25 × 50		
	CuSO ₄ ·5H ₂ O	0.025 × 50		
	CoCl ₂ ·6H ₂ O	0.025 × 50		
D	Iron-EDTA		100	10
	Na ₂ EDTA·2H ₂ O	37.3 × 10		
	FeSO ₄ ·7H ₂ O	27.8 × 10		
E	Vitamins			
	Glycine	2.0 × 100	100	1
	Nicotinic Acid	0.5 × 100		
	Pyridoxine-HCl	0.5 × 100		
	Thiamine-HCl	0.1 × 100		
F	Myo-Inositol			
	Myo-Inositol	100 × 10	100	10
	Sucrose			
	Agar	30,000	Added directly	Added directly
	pH	7,000 5.8		

Table 3.3 Details of the hormones used for tissue culture

Hormone	Purpose	Working concentration	Stock	Supplier
Benzyl amino purine (BAP)	For shoot multiplication and elongation	0.2-0.5 mg /l	1 mg/ml	Sigma
Naphthalene acetic acid (NAA)	For shoot induction and root induction	0.2 mg /l	1 mg/ml	Sigma
Thidiazuron (TDZ)	For shoot induction	0.2 mg /l	1 mg/ml	Sigma
Kinetin	For elongation	1-2 mg /l	1 mg/ml	Sigma
Indole-3 butyric acid (IBA)	For root induction	1-2 mg /l	1 mg/ml	Sigma
Phloroglucinol	For root induction	1-2 µg/ml	1 mg/ml	Sigma

Table 3.4 Details of the antibiotics/herbicide used for tissue culture

Antibiotic/ hormone/ herbicide	Purpose	Working concentration (mg/ml)	Stock (mg/ml)	Supplier
Ampicillin	For selection of ampicillin resistant <i>E. coli</i> strains	100	100	USB
Kanamycin	For selection of binary vector	50	50	Duchefa
Streptomycin	Marker for helper plasmid of <i>Agrobacterium</i>	25	25	Synbiotics
Rifampicin	Marker for chromosome of <i>Agrobacterium</i>	25	25	Duchefa
Hygromycin	Transgenic plant selection	5-20	20	Duchefa
Cefotaxime	Elimination of <i>Agrobacterium</i>	250	250	Alkem
Phosphinothricin	Transgenic plant selection	0.5-1.0	10	Duchefa

3.1.6 Other chemicals and consumables

The chemicals and kits used were of molecular biology grade and obtained from Sigma, Amersham Biosciences, USB, Bangalore Genei, Life Technologies, Genetix, Himedia, Invitrogen, MBI Fermentas, Qiagen, Ducheфа, Sigma, etc. Other consumables like plasticware, glassware, scalpel blades, filter papers, forceps etc., were from Tarsons, Axygen, Borosil companies. Standard solutions and buffers were prepared according to the procedures given by Ausubel *et al.* (1999).

3.1.7 Vectors and Strains

Salient features of the commercial vectors used in the present study are given in Table 3.5. The physical maps and the features of T/A, pUC18, pRT100, pCAMBIA0390 and pCAMBIA1300 vectors are shown in Figures 3.1, 3.2, 3.3, 3.4, 3.5. Other recombinant vectors utilized for the present study are pBARGUS, pCAMBIA1305.2-TCON and pRT-Cat Int (Table 3.6; Figures 3.6, 3.7 and 3.8). Standard bacterial strain DH5 α of *Escherichia coli* was used for maintenance of all the recombinant plasmids. The developed recombinant vectors were mobilized into *Agrobacterium tumefaciens* strain LBA4404, which was further used for plant transformation.

3.2 Methods

3.2.1 Development of gene constructs

3.2.1.1 Primer designing

The aim was to identify mitochondrial genes based on their RNA editing status and subsequently develop constructs for induction of male sterility as well as for fertility

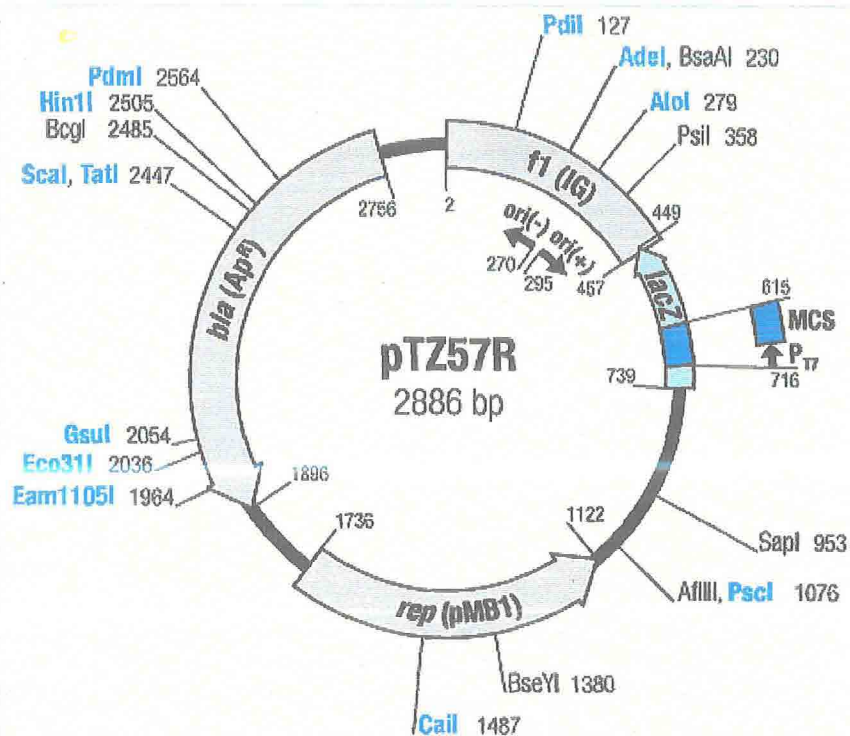


Fig. 3.1a Schematic diagram of T/A cloning vector (MBI Fermentas)

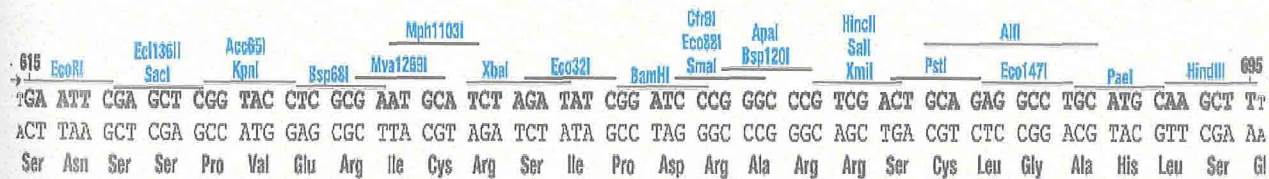


Fig. 3.1b Multiple Cloning Sites (MCS) of T/A vector (MBI Fermentas)

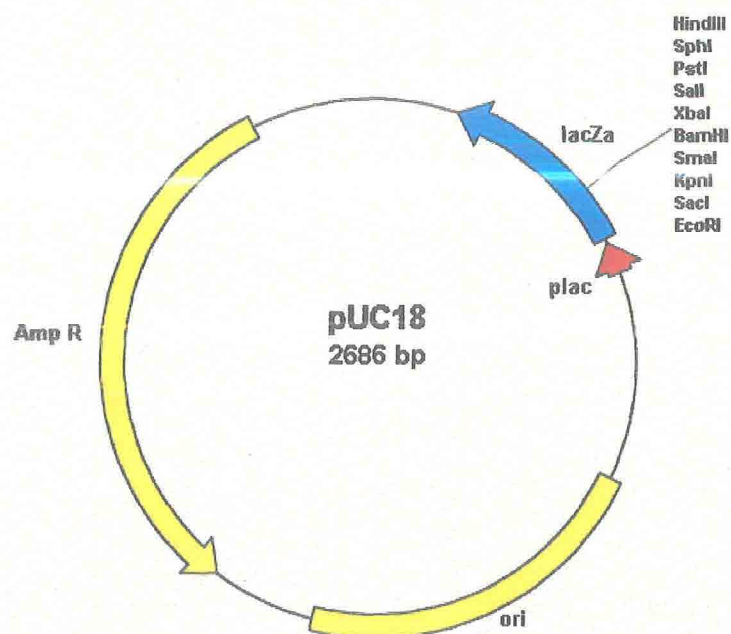


Fig. 3.2 Schematic diagram of pUC18 vector

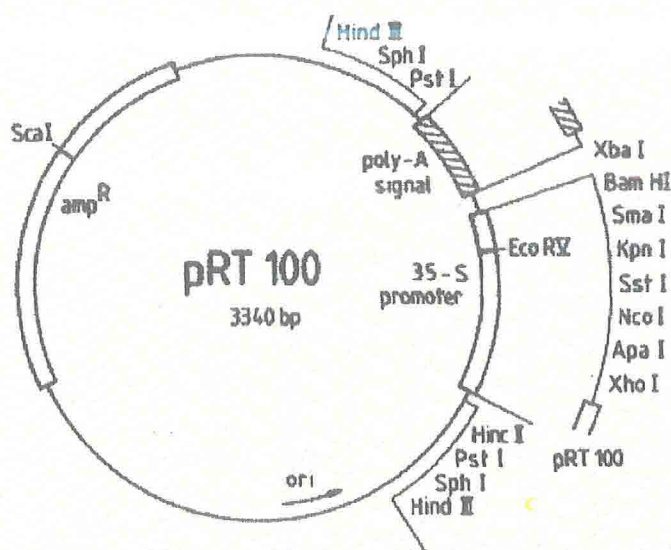


Fig. 3.3 Schematic diagram of pRT100 vector

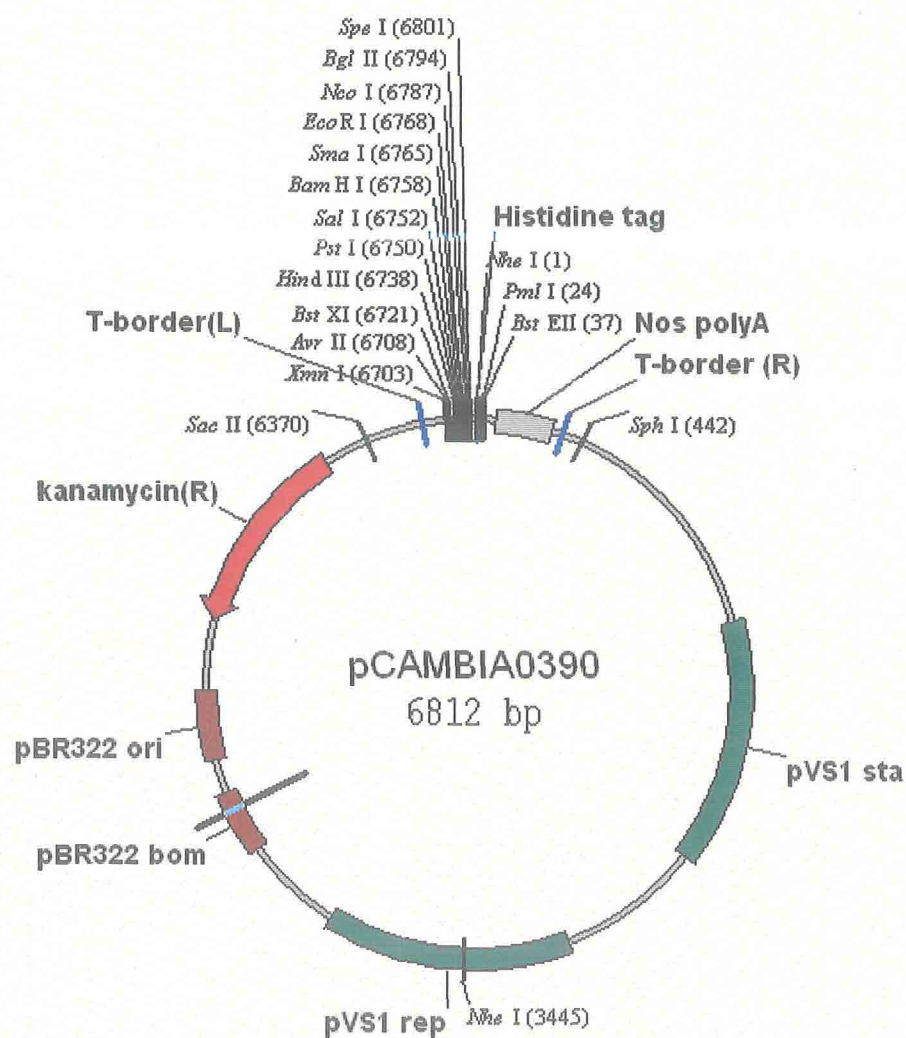


Fig. 3.4 Plasmid map of pCAMBIA0390 vector

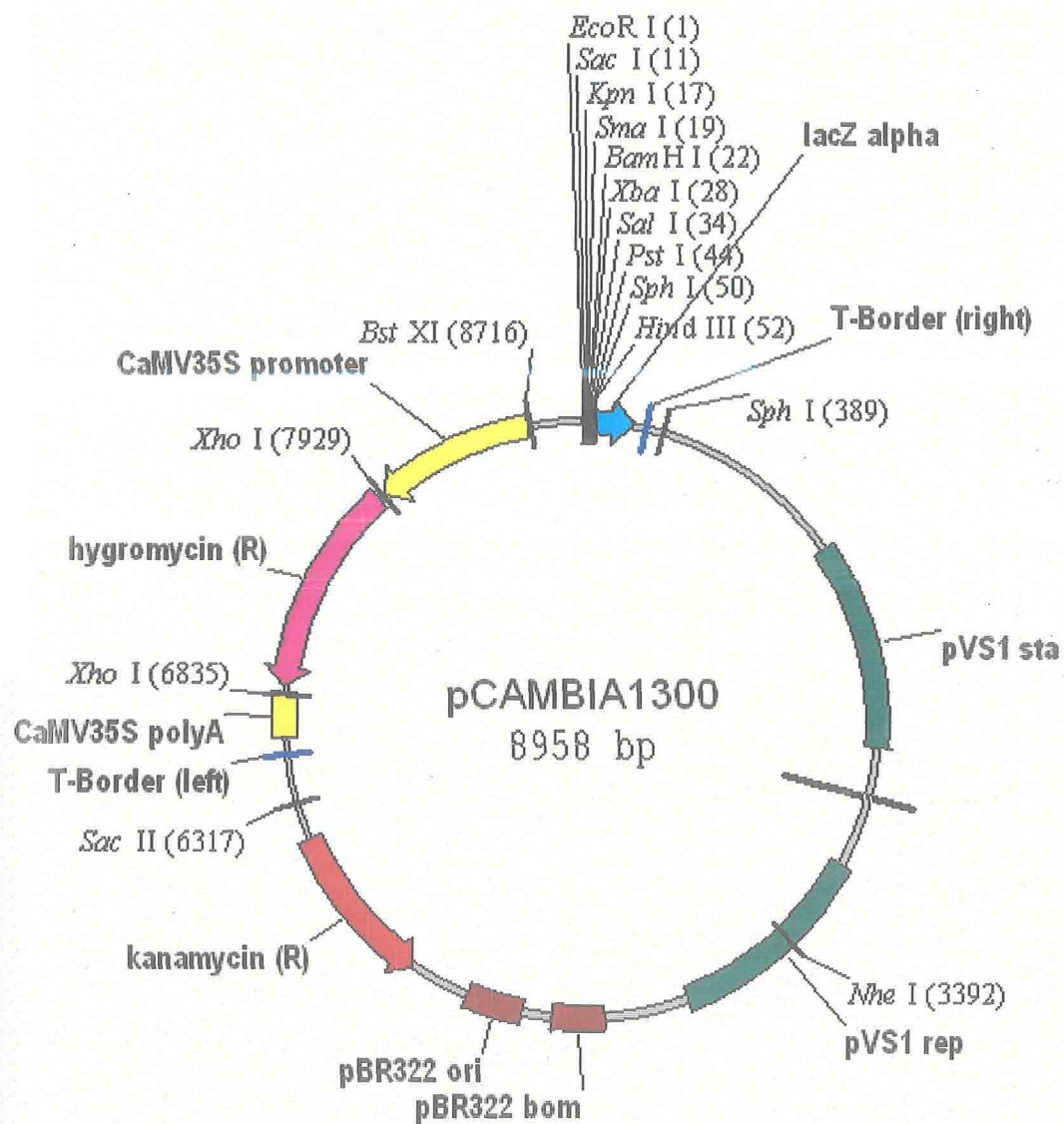


Fig. 3.5 Plasmid map of pCambia1300 vector

Table 3.5 Salient features of the commercial vectors used

Plasmid	Size (bp)	Multiple cloning site (MCS)	Bacterial selection marker	Blue/white selection (<i>lacZ</i>)	Purpose of use
pUC18	2686	<i>HindIII</i> , <i>SphI</i> , <i>SdaI</i> , <i>PstI</i> , <i>BspMI</i> , <i>SalI</i> , <i>XbaI</i> , <i>BamHI</i> , <i>SmaI</i> , <i>KpnI</i> , <i>SacI</i> , <i>EcoRI</i>	Ampicillin	Present	Preliminary cloning of <i>Bar</i> gene cassette taken from pBARGUS
pRT100	3340	<i>XhoI</i> , <i>ApaI</i> , <i>NcoI</i> , <i>SstI</i> , <i>KpnI</i> , <i>SmaI</i> , <i>BamHI</i> , <i>XbaI</i>	Ampicillin	Absent	Preliminary development of all fertility restoration constructs
pTZ57R T/A	2886	<i>EcoRI</i> , <i>SacI</i> , <i>KpnI</i> , <i>Bsp681</i> , <i>MphI</i> , <i>XbaI</i> , <i>BamHI</i> , <i>SmaI</i> , <i>SmaI</i> , <i>ApaI</i> , <i>SalI</i> , <i>EcoI</i> , <i>PaeAI</i> , <i>HindIII</i>	Ampicillin	Present	Initial cloning of <i>u-atp6</i> , <i>u-nad3</i> , <i>u-atp9</i> (genes and cDNAs)
pCAMBIA1305.2	11921	<i>EcoRI</i> , <i>SacI</i> , <i>KpnI</i> , <i>SmaI</i> , <i>BamHI</i> , <i>XbaI</i> , <i>SalI</i> , <i>PstI</i> , <i>SphI</i> , <i>HindIII</i>	Kanamycin	Present	Initial cloning of <i>u-nad3</i> and <i>u-atp9</i> in pCAMBIA1305.2-TCO to develop TCNN and TCAN
pCAMBIA1300	8958	<i>EcoRI</i> , <i>SacI</i> , <i>KpnI</i> , <i>SmaI</i> , <i>BamHI</i> , <i>XbaI</i> , <i>SalI</i> , <i>PstI</i> , <i>SphI</i> , <i>HindIII</i>	Kanamycin	Absent	For cloning the gene cassettes developed for restoration of fertility
pCAMBIA0390	6812	<i>XmnI</i> , <i>BstXI</i> , <i>HindIII</i> , <i>PstI</i> , <i>SalI</i> , <i>BamHI</i> , <i>SmaI</i> , <i>EcoRI</i> , <i>NcoI</i> , <i>BalII</i> , <i>SpeI</i> , <i>NheI</i> , <i>PmlI</i> , <i>BstEII</i>	Kanamycin	Absent	For cloning the gene cassettes developed for induction of male sterility

Table 3.6 Other recombinant vectors utilized for cloning purpose

Vector	Components	Bacterial selection marker	Reference
pCAMBIA1305.2-TCO	TA29 promoter, <i>coxIV</i> Presequence, <i>orfH522</i> gene, <i>nos</i> terminator	Kanamycin	Harinath <i>et al.</i> , 2004
pRT100-Cat Int	35S promoter, catalase intron, poly A terminator	Ampicillin	Ashraf <i>et al.</i> , 2007
pBARGUS	35S promoter, <i>bar</i> gene, poly A terminator	Kanamycin	Prof. Rong Du, USA

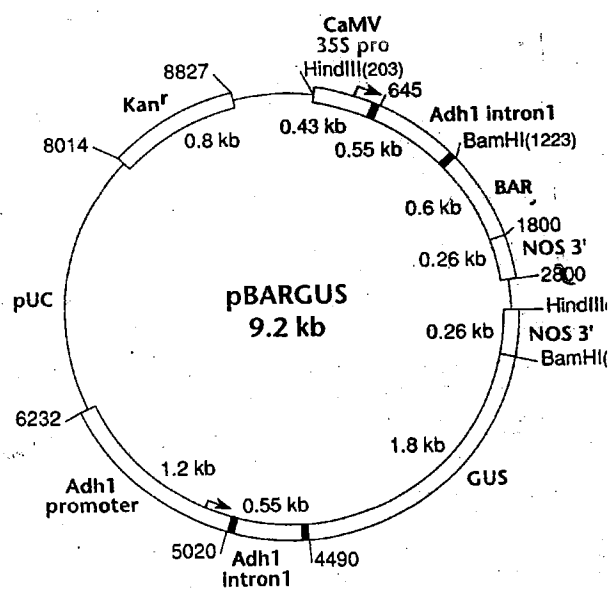


Fig. 3.6 Map of pBARGUS vector

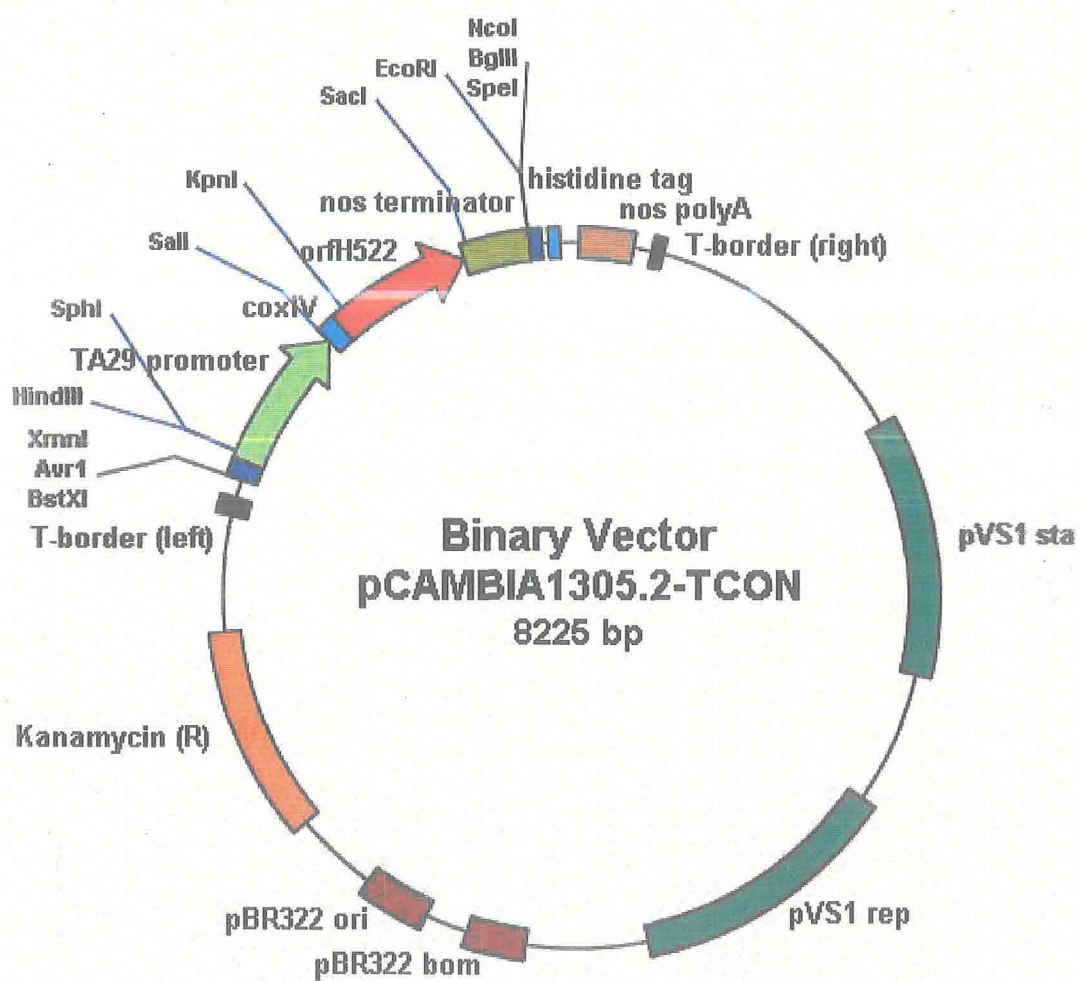


Fig. 3.7 Plasmid map of pCambia1305.2-TCON

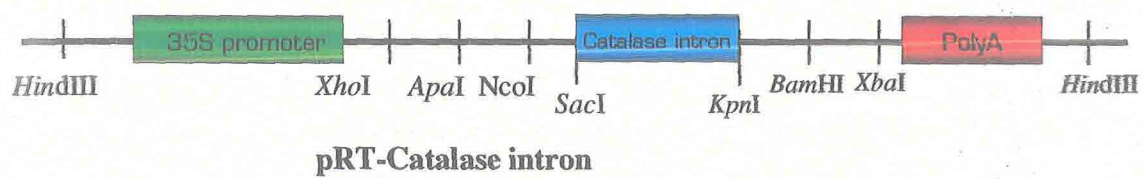


Figure 3.8 Map of the generic vector pRT100-Cat Int

restoration based on these genes. Primers for the genes *atp6*, *nad3* and *atp9* were designed based on their sequences in sunflower available at National Center for Biological Information website: www.ncbi.nlm.nih.gov due to the non-availability of sequence information regarding mitochondrial genes in safflower. Also, sunflower as well as safflower belong to the same family (Asteraceae) and hence are expected to share a high level of sequence homology. Due to this reason, sunflower nucleic acids (DNA/RNA) were used as control in most of the PCR reactions. Table 3.7 gives the details of the sequences isolated and utilized in the present study. Several primer sets were designed and used for the present investigation. Some primers available already at DOR were also used (Table 3.8).

While designing primers, care was taken to add appropriate restriction enzyme sites so as to facilitate the directional cloning of the component sequences for each of the constructs developed. Restriction enzyme sites that were introduced at the 5' end of the primers for *nad3* and *atp9* were *KpnI*, *SacI*, *BamHI* and *XhoI*. To enable the PCR product to be cut by the cloning enzymes, a few (2-6) bases of buffer sequence were introduced before these sites as per the guidelines provided by the supplier of the enzymes (Genei/MBI Fermentas).

For identification of suitable mitochondrial genes during the present study, all the three genes *atp6*, *nad3* and *atp9* were amplified from safflower by PCR and RT-PCR for developing the genomic and cDNA clones of these genes. During the development of constructs for induction of male sterility, the *u-nad3* and *u-atp9* genes (from the genomic clones) were cloned into the pCAMBIA1305.2-TCON (pCAMBIA1305.2:TA29-CoxIV-*OrfH522-nos* terminator) vector that was earlier developed at DOR. The primers were designed with appropriate restriction enzyme sites so that *orfH522* could be replaced with

Table 3.7 Details of the sequences isolated and/or utilized

Sequences	Source	GenBank accession number	Nucleotides (as in GenBank)	Size (bp)	Flanking enzymes	Expected PCR product (bp)	Used for
<i>atp6</i> gene	<i>Helianthus annuus</i>	X82387	1-906	906	-	906	Designing primers for use in safflower
<i>nad3</i> gene	<i>Helianthus annuus</i>	AF258785	1-357	357	-	357	Designing primers for use in safflower
<i>atp9</i> gene	<i>Helianthus annuus</i>	X84008	1-261	261	-	261	Designing primers for use in safflower
<i>nad3</i> gene	<i>Carthamus tinctorius</i>	DQ539623*	1-357	357	<i>KpnI, SacI</i>	372	Determined in the present study and used for developing constructs
<i>atp9</i> gene	<i>Carthamus tinctorius</i>	DQ539624*	1-261	261	<i>KpnI, SacI</i>	277	Determined in the present study and used for developing constructs
<i>nad3</i> RNA	<i>Carthamus tinctorius</i>	DQ534204*	1-357	357	<i>KpnI, SacI</i>	372	Determined in the present study
<i>atp9</i> mRNA	<i>Carthamus tinctorius</i>	DQ534203*	1-261	261	<i>KpnI, SacI</i>	277	Determined in the present study

* These sequences were obtained in the present study and have been submitted to GenBank

gene of interest in the present investigation. All the primers used in the present study along with their details are given in Table 3.8. The T_m values of primers were calculated using the formula as given by Sambrook and Russell (2001).

$$T_m = 2 (A+T) + 4 (G+C)$$

3.2.1.2 Nucleic acid isolation

Initially total DNA, mitochondrial DNA and mitochondrial RNA were isolated from sunflower and safflower. As all the primer sequences were designed based on the sunflower sequences, sunflower was used as a control during studies on RNA editing. During transformation and generation of transgenic plants, tobacco being a good model system (with easy regeneration and transformation) was used as a control with the presumption that this system would facilitate easier and quicker validation of the constructs that have been developed in the present study. For confirmation of transgenic tobacco and safflower plants, their genomic DNA isolations were also carried out.

3.2.1.2.1 Genomic DNA isolation

DNA was isolated by using the modified CTAB procedure according to Doyle and Doyle (1987). Fresh leaves (2g) were ground to fine powder in liquid nitrogen using pre-chilled mortar and pestle. The homogenized powder was transferred to 15 ml of pre-warmed (60°C) isolation buffer [2% (w/v) CTAB, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris-HCl (pH 8.0), 0.2% β -mercaptoethanol (added just before use)] and incubated at 60°C for 30-60 minutes. The solution was extracted twice with equal volume of chloroform-isoamylalcohol (24:1). The final aqueous phase was transferred to a fresh tube and DNA was precipitated with 0.6

Table 3.8 Details of the primers used in the present study

S#	Primer	Length	T _m	Sequence
1	<i>Kpn</i> I <i>atp6</i> F I	33 bp	64	5' atg gta cca tga gag tag taa agg att att atg 3'
2	<i>Kpn</i> I <i>atp6</i> F II	32 bp	64	5' at ggt acc tcc cat tga ttc cta tga ata tag 3'
3	<i>Kpn</i> I <i>atp6</i> F III	32 bp	64	5' at ggt acc ctt gta ttt ctc att cac aaa ttc 3'
4	<i>Sac</i> I <i>atp6</i> R I	32 bp	64	5' a gag ctc tta atg gag att tat agc atc att c 3'
5	<i>Sac</i> I <i>atp6</i> R II	29 bp	64	5' a gag ctc ttt gtg aat gag aaa tac aag t 3'
6	<i>Sac</i> I <i>atp6</i> R III	30 bp	62	5' a gag ctc gac ccc cta ttt gtt cgt tta cc 3'
7	<i>Kpn</i> I <i>nad3</i> F2	31 bp	59	5' at ggt acc atg tca gaa ttt gca cct att tg 3'
8	<i>Sac</i> I <i>nad3</i> R2	27 bp	63	5' a gag ctc tta ctc ccg atc cga agc ac 3'
9	<i>nad3</i> F1	20 bp	62	5' caa agt ggg ctg tgg tga tg 3'
10	<i>nad3</i> R1	20 bp	60	5' cct atc gct agt ggt tac tc 3'
11	<i>Kpn</i> I <i>atp9</i> F2	33 bp	59	5' at ggt acc atg tta gaa ggt gca aaa cta ata g 3'
12	<i>Sac</i> I <i>atp9</i> R2	30 bp	58	5' a gag ctc tta tga aac ttc aac ctt ctt tc 3'
13	<i>atp9</i> F1	21 bp	60	gag aat gat caa ctc gag atg
14	<i>atp9</i> R1	23 bp	62	tgc ttg ctt tat gaa act tca ac
15	<i>Sph</i> I TA29 F*	33 bp	59	5' tac atg cat gca tct agc taa gta taa ctg gat 3'
16	<i>Sal</i> I TA29 R*	35 bp	60	5' act gtc gac ggt agc taa ttt ctt taa gta aaa ac 3'
17	<i>Sal</i> I <i>coxIV</i> F*	63 bp	70	5' act gtc gac atg ctt tca cta cgt caa tct ata aga ttt ttc aag cca gcc aca aga act ttg 3'
18	<i>Kpn</i> I <i>coxIV</i> R*	54 bp	71	5' atg gta ccc tga agc aga tat cta gag cta cac aaa gtt ctt gtg gct ggc ttg 3'
19	<i>Sac</i> I <i>nos ter</i> F*	27 bp	60	5' a gag ctc cga agc aga tgg ttc aaa ca 3'
20	<i>Eco</i> RI <i>nos ter</i> R*	30 bp	59	5' gga att ctc ccg atc tag taa cat aga tga 3'

Table 3.8(cont.).

S#	Primer	Length	T _m	Sequence
21	pRT100 Seq*	23 bp	64	5' cct tcc tct ata taa gga agt tc 3'
22	<i>Sst</i> I Cat Int F*	30 bp	58	5' aga gct cag ggt aaa ttt cta gtt ttt ctc 3'
23	<i>Kpn</i> I Cat IntR*	31 bp	59	5' atg gta ccg ttc tgt aac tat cat cat cat c 3'
24	<i>Hinc</i> II TA29 F	28 bp	59	5' gtc aac atc tag cta agt ata act gga t 3'
25	<i>Xho</i> I TA29 R	32 bp	60	5' ctc gag ggt agc taa ttt ctt taa gta aaa ac 3'
26	<i>Sst</i> I PolyA F*	27 bp	62	5' aga gct cgt ccg caa atc acc agt ctc 3'
27	<i>Nco</i> I PolyAR*	29 bp	59	5' atc cat ggg tca ctg gat ttt ggt ttt ag 3'
28	<i>Kpn</i> I <i>Sac</i> I <i>nad3</i> F	37 bp	64	5' aa ggt acc gag ctc atg tca gaa ttt gca cct att tg 3'
29	<i>Bam</i> HI <i>Xho</i> I <i>nad3</i> R	33 bp	68	5' aa gga tcc ctc gag tta ctc ccg atc cga agc ac 3'
30	<i>Kpn</i> I <i>Sac</i> I <i>atp9</i> F	39 bp	64	5' aa ggt acc gag ctc atg tta gaa ggt gca aaa cta ata g 3'
31	<i>Bam</i> HI <i>Xho</i> I <i>atp9</i> R	36 bp	63	5' aa gga tcc ctc gag tta tga aac ttc aac ctt ctt tc 3'

* These primers were designed previously but utilized for the present investigation.

All other primers were designed during this study

volumes of ice-cold isopropanol. The DNA pellet obtained after centrifugation was washed with 70% ethanol, air-dried and dissolved in 100 μ l T₁₀E₁ buffer [10 mM Tris-HCl, 1 mM EDTA (pH 8.0)].

3.2.1.2.2 Isolation of mitochondria and Mitochondrial DNA

Mitochondria were isolated following the protocol of Kirti *et al.* (1992). Unopened young florets collected from inflorescences were separated with a sterile blade. 20 g of florets were homogenized in 5-7 volumes of ice-chilled buffer A [10 mM Tris-HCl (pH 7.2), 1 mM EDTA (pH 7.2), 0.5 M mannitol, 0.2% BSA, 0.05% cysteine]. The homogenized solution was filtered and spun at 3000 rpm (Heraeus) for 10 min (4°C). The supernatant was separated and spun at 12000 rpm for 10 min (4°C). The pellet was resuspended in 10 ml of buffer A with the help of a camel hair brush and centrifuged at 4000 rpm for 15 min (4°C). To the supernatant, MgCl₂ (10 mM final concentration) and DNase (10 μ g/g tissue) were added and incubated on ice for 1 h. This solution was overlaid on 2 volumes of buffer B [10 mM Tris (pH 7.2), 20 mM EDTA (pH 7.2), 0.6 M sucrose] and spun at 12000 rpm for 10 min (4°C). The mitochondrial pellet obtained was washed twice with buffer B and used for mt DNA and mt RNA isolation.

For DNA isolation, the mitochondrial pellet was resuspended in lysis buffer [10 mM Tris (pH 8.0), 10 mM EDTA (pH 8.0), 2% sarcosine], proteinase K was added at 0.012% and incubated at 37°C for 1 h. The reaction was terminated with 0.3 M sodium acetate (pH 5.2). The supernatant was extracted with an equal volume of phenol: chloroform: isoamylalcohol (25:24:1) and twice with chloroform: isoamylalcohol (24:1) and the DNA was precipitated

with absolute ethanol in excess of 2.5 times the volume of solution. The DNA pellet was washed with 70% ethanol, air dried and dissolved in 50 μ l T₁₀E₁ buffer.

3.2.1.2.3 Mitochondrial RNA isolation

To obtain a good preparation of RNA, RNase activity was minimized by following the guidelines as given by Sambrook and Russell, 2001.

- Beakers, tubes and other items that were used for the RNA were filled with diethyl pyrocarbonate (DEPC) (0.1% in water), rinsed several times with sterile water and autoclaved for 40 minutes at 103.5 kPa.
- Electrophoresis tank was cleaned with detergent solution, rinsed with water, dried with ethanol and then filled with a solution of 3% H₂O₂ and allowed to stand for 10 minutes and rinsed thoroughly with autoclaved water that was treated with 0.1% DEPC.
- Disposable plastic-wares like eppendorf tubes, pipette tips, PCR tubes were autoclaved for 40 minutes at 121°C (103.5 kPa)
- All the solutions were prepared using RNase-free glassware, 0.1% DEPC-treated water and autoclaved for 30 minutes at 103.5 kPa.
- Contamination from hands was avoided by using disposable powder-free gloves.

Total RNA from the mitochondria of the male florets was extracted using TRIzol[®] according to the protocol given by the supplier (Life Technologies). The mitochondrial pellet was dissolved in TRIzol[®] reagent (@1ml per 50-100mg of tissue) and spun at 12000g(Heraeus) for 10minutes. The clear homogenate was vigorously shaken with chloroform (1/5th volume of the TRIzol[®] used) and incubated at room temperature (15-30°C) for 2-3 minutes and spun at 12000 g (Heraeus) for 15 min (4°C). The aqueous phase was transferred into a fresh tube

and RNA was precipitated with 0.25 ml of isopropanol, 0.25 ml of high salt precipitation solution (1.2 M sodium chloride and 0.5 M sodium citrate) per 1 ml of TRIzol[®] used. 40 units of RNase inhibitor was added to overcome RNase activity. The RNA was pelleted by centrifugation at 12000 g (Heraeus) for 10 min. The pellet was washed with 75% ethanol (prepared with autoclaved DEPC treated water), air dried and dissolved in T₁₀E₁ buffer.

DNase treatment for RNA: The procedure as given by the supplier of DNase (Ambion) was followed. The reaction consisted of 1-2 µg of RNA, 2 units of DNase and the reaction buffer (1x final concentration) incubated at 37°C for 30 min. The reaction was stopped with 5 µl of DNase inactivation reagent. The mixture was spun at 10000 g (Heraeus) for one minute to pellet the DNase inactivation reagent.

3.2.1.2.4 Plasmid DNA isolation

Plasmid DNA of required plasmid/vectors was isolated from 3 ml of overnight grown culture. The bacterial cells were spun down at 12000 g for 1 min. The pellet was resuspended in 100 µl sterile double distilled water. 100 µl of lysis buffer (1% SDS, 10 mM EDTA, 0.1 N NaOH of final concentration) was added and boiled at 85-100°C for 2 min. This was followed by addition of 50 µl of 1.0 M MgCl₂ and incubation on ice for 2 min and centrifugation at 12000 g for 2 min. To the same tubes, 50 µl of 5 M potassium acetate (pH 5.2) was added, mixed by tapping and left on ice for 2 min and spun at 12000 g for 2 min. The supernatant was transferred to fresh tubes and the DNA was precipitated with absolute ethanol and pelleted by centrifugation. The pellet was washed with 70% ethanol, air dried and dissolved in 50 µl T₁₀E₁ buffer. For sequencing purpose, the plasmid DNA from

selected clones was isolated using QIAprep® Spin Miniprep Kit (Qiagen) as per the suppliers' protocol.

3.2.1.3 PCR amplification

During development of constructs, whenever the inserts were isolated by PCR (Table 3.9), a preparative PCR (10 or 20 µl reaction volume) was carried out with primers specific to each of the components with the respective DNA or RNA. Once the expected size fragment was obtained a large volume PCR (60 or 80 µl reaction volume) was carried out with the same PCR conditions. The PCR reaction mixture consisted of 6-10 ng of DNA, 0.2 µM each of the forward and reverse primers, 100 µM of each dNTP, 1x concentration of *Taq* DNA polymerase buffer (10 mM Tris-HCl (pH 9.0), 1.5 mM MgCl₂, 50 mM KCl, 0.01% gelatin), 1 unit of *Taq* DNA polymerase and milliQ water to a total volume of 60 µl. The product thus generated was used for cloning. The clones generated were confirmed by PCR and/or restriction digestion. Confirmation of the transgenic plants was done using PCR. Appropriate primers for confirming the transgene constructs were utilized in the PCR reaction. The general PCR program utilized for this study is given below. Only the annealing temperature (based on T_m of the primers) and/or the extension time varied (according to the length of the amplicons). The cycling parameters adopted for PCR were:

Step	Temperature (°C)	Time	Cycles
Initial denaturation	92	3 min	1
Denaturation	92	30 sec	30
Annealing	58-60	30 sec	
Extension	72	25 sec-1min 30 sec	
Final extension	72	7 min	1
Hold	4	∞	-

Table 3.9 Details of the clones developed in the present study

Insert	Source for insert isolation	Method of insert isolation	Vector into which insert was cloned	Enzymes used for cloning	Method of confirmation	Size of insert	Clones generated
Development of constructs for induction of transgenic male sterility							
Genomic (unedited) <i>nad3</i>	Safflower mt DNA	PCR	T/A	-	PCR and Restriction Digestion	357 bp	T/A- <i>nad3</i>
Genomic (unedited) <i>atp9</i>	Safflower mt DNA	PCR	T/A	-	PCR and Restriction Digestion	261 bp	T/A- <i>atp9</i>
Genomic (unedited) <i>nad3</i>	T/A- <i>nad3</i>	PCR	pCAMBIA1305.2-TCNN	<i>KpnI</i> , <i>SacI</i>	PCR and Restriction Digestion	357 bp	pCAMBIA1305.2-TCNN
Genomic (unedited) <i>atp9</i>	T/A- <i>atp9</i>	PCR	pCAMBIA1305.2-TCNN	<i>KpnI</i> , <i>SacI</i>	PCR and Restriction Digestion	261 bp	pCAMBIA1305.2-TCAN
TCNN	pCAMBIA1305.2-TCNN	Restriction Digestion	pCAMBIA0390	<i>HindIII</i> , <i>EcoRI</i>	PCR and Restriction Digestion	1.38 kb	pCAMBIA0390-TCNN
TCAN	pCAMBIA1305.2-TCAN	Restriction Digestion	pCAMBIA0390	<i>HindIII</i> , <i>EcoRI</i>	PCR and Restriction Digestion	1.18 kb	pCAMBIA0390-TCAN
<i>Bar</i> construct	pBARGUS	Restriction Digestion	pUC18	<i>HindIII</i>	Restriction Digestion	2.1 kb	pUC18- <i>Bar</i>
<i>Bar</i> construct	pUC18- <i>Bar</i>	Restriction Digestion	pCAMBIA0390-TCNN	<i>HindIII</i>	Restriction Digestion	2.1 kb	pBin-TCNN- <i>Bar</i> *
<i>Bar</i> construct	pUC18- <i>Bar</i>	Restriction Digestion	pCAMBIA0390-TCAN	<i>HindIII</i>	Restriction Digestion	2.1 kb	pBin-TCAN- <i>Bar</i> *

Table 3.9 (cont.).

Development of constructs for restoration of fertility							
Insert	Source of insert isolation	Method of insert isolation	Vector into which insert was cloned	Enzymes used for cloning	Method of confirmation	Size of insert	Clones generated
Genomic (unedited) <i>nad3</i>	T/A- <i>nad3</i>	Restriction Digestion	pRT100	<i>Kpn</i> I, <i>Sac</i> I	Restriction Digestion	357 bp	p-SANP
Genomic (unedited) <i>atp9</i>	T/A- <i>atp9</i>	Restriction Digestion	pRT100	<i>Kpn</i> I, <i>Sac</i> I	Restriction Digestion	261 bp	p-SAAP
SANP	p-SANP	Restriction Digestion	pCAMBIA1300	<i>Hind</i> III	Restriction Digestion	1.1 kb	pCAMBIA1300-SANP
SAAP	p-SANP	Restriction Digestion	pCAMBIA1300	<i>Hind</i> III	Restriction Digestion	1.0 kb	pCAMBIA1300-SAAP
TA29	T/A	Restriction Digestion	p-SANP	<i>Hinc</i> II, <i>Xho</i> I	Restriction Digestion	545 bp	p-TANP
TA29	T/A	Restriction Digestion	p-SAAP	<i>Hinc</i> II, <i>Xho</i> I	Restriction Digestion	545 bp	p-TAAP
TANP	p-TANP	Restriction Digestion	pCAMBIA1300	<i>Hind</i> III	Restriction Digestion	1.2 kb	pCAMBIA1300-TANP
TAAP	p-TAAP	Restriction Digestion	pCAMBIA1300	<i>Hind</i> III	Restriction Digestion	1.1 kb	pCAMBIA1300-TAAP
Genomic (unedited) <i>nad3</i>	Safflower mt DNA	PCR	T/A	-	PCR and Restriction Digestion	357 bp	T/A- <i>nad3</i> (ihp)
Genomic (unedited) <i>atp9</i>	Safflower mt DNA	PCR	T/A	-	PCR and Restriction Digestion	261 bp	T/A- <i>atp9</i> (ihp)
Antisense <i>u-nad3</i>	T/A- <i>nad3</i> (ihp)	Restriction Digestion	pRT100-CI	<i>Xho</i> I, <i>Sac</i> I	Restriction Digestion	357 bp	p-CI-Antisense <i>nad3</i>
Antisense <i>u-atp9</i>	T/A- <i>atp9</i> (ihp)	Restriction Digestion	pRT100-CI	<i>Xho</i> I, <i>Sac</i> I	Restriction Digestion	261 bp	p-CI-Antisense <i>atp9</i>
Sense <i>u-nad3</i>	T/A- <i>nad3</i> (ihp)	Restriction Digestion	p-CI-Antisense <i>nad3</i>	<i>Kpn</i> I, <i>Bam</i> HI	Restriction Digestion	357 bp	p-ihp <i>nad3</i>
Sense <i>u-atp9</i>	T/A- <i>atp9</i> (ihp)	Restriction Digestion	p-CI-Antisense <i>atp9</i>	<i>Kpn</i> I, <i>Bam</i> HI	Restriction Digestion	261 bp	p-ihp <i>atp9</i>
ihp <i>nad3</i>	p-ihp <i>nad3</i>	Restriction Digestion	pCAMBIA1300	<i>Hind</i> III	Restriction Digestion	1.6 kb	pBin-ihp <i>nad3</i> *

Table 3.9(cont.).

Insert	Source of insert isolation	Method of insert isolation	Vector into which insert was cloned	Enzymes used for cloning	Method of confirmation	Size of insert	Clones generated
ihp <i>atp9</i>	p-ihp <i>atp9</i>	Restriction Digestion	pCAMBIA1300	<i>HindIII</i>	Restriction Digestion	1.5 kb	pBin-ihp <i>atp9</i> *
TA29	T/A-TA29	Restriction Digestion	pRT100	<i>HincII</i> , <i>XhoI</i>	Restriction Digestion	525 bp	p-TA29
ihp <i>nad3</i>	p-ihp <i>nad3</i>	Restriction Digestion	p-TA29	<i>XhoI</i>	Restriction Digestion	940 bp	p-T-ihp <i>nad3</i>
ihp <i>atp9</i>	p-ihp <i>atp9</i>	Restriction Digestion	p-TA29	<i>XhoI</i>	Restriction Digestion	742 bp	p-T-ihp <i>atp9</i>
T-ihp <i>nad3</i>	p-T-ihp <i>nad3</i>	Restriction Digestion	pCAMBIA1300	<i>PstI</i>	Restriction Digestion	1.6 kb	pBin-T-ihp <i>nad3</i> *
T-ihp <i>atp9</i>	p-T-ihp <i>atp9</i>	Restriction Digestion	pCAMBIA1300	<i>PstI</i>	Restriction Digestion	1.5 kb	pBin-T-ihp <i>atp9</i> *

* These clones were mobilized into *Agrobacterium* strain LBA4404

3.2.1.4 Reverse Transcription-PCR

RT-PCR was employed to synthesize cDNA of *atp6*, *nad3* and *atp9* and to develop cDNA clones in order to determine the RNA editing status of these genes. Also, RT-PCR with *atp6* RII and *atp6* RIII primers was done in order to determine the sequence on the 5' end of *atp6* cDNA (by employing 5'RACE method). The first-strand cDNA synthesis was carried out as per the instructions given by the supplier (Invitrogen). The first-strand cDNA synthesis reaction mixture consisted of 1 µg of total RNA (DNase treated), 2 µM each of the reverse primer (*SacI atp6* R I, *SacI nad3* R and *SacI atp9* R were used during RT reaction for cDNA synthesis of *atp6*, *nad3* and *atp9* respectively), 1x final concentration of reaction buffer [20 mM Tris-HCl (pH 8.4), 50 mM KCl], 0.1M dithiothreitol (DTT), 40 units of recombinant ribonuclease inhibitor (RNase OUT), 25 mM MgCl₂, 100 µM of each dNTP, 50 units of SuperscriptTM II RT enzyme and sterile water to make up the volume to 30 µl. During the first step, RNA, primer, dNTP and sterile water were incubated at 70°C for 10 min and immediately brought to 4°C in a thermal cycler. To this, a mixture containing RT-buffer, DTT, RNaseOUT, MgCl₂ and SuperscriptTM enzyme was added and reverse transcription was carried out on thermal cycler by adopting the following profile:

45°C for 50 minutes

70°C for 15 minutes

4°C hold

The resultant cDNA was used for PCR amplifications with the same conditions and programme as mentioned in section 3.2.3.

3.2.1.5 5' - Rapid Amplification of cDNA Ends (RACE)

Though the expected size of *atp6* cDNA was 906 bp (obtained in case of sunflower), only a band of ~250 bp was obtained with safflower during RT-PCR. Since the 5' portion of *atp6* was different in safflower compared to sunflower, it was intended to determine this additional sequence in *atp6* cDNA by employing the 5' RACE technique. The RT-product (resultant from 1-2 µg of safflower RNA) was treated with RNase H (incubated with 1 µl of the enzyme at 37°C for 20 min) and then PCR purified using the Qiagen PCR purification kit. A poly G oligonucleotide was ligated to the 3'OH end of the cDNA molecule using T₄ DNA ligase (Fermentas). This reaction included 1ng of the above PCR purified cDNA (after RNase H treatment), 1x final concentration of the 10x buffer (supplied along with the enzyme with components: 500 mM HEPES-NaOH (pH 8.0 at 25°C), 100 mM MgCl₂, 100 mM DTT), 5 units of T₄ DNA ligase (Fermentas) and 0.2 µM final concentration of the poly G oligonucleotide. This mixture was incubated at 37°C for 1 hr. 1 µl of this product (polyG ligated cDNA) was used to set up a PCR reaction (the components and conditions for PCR reaction were as mentioned above), using *atp6* RIII and polyC primers. The resultant amplicons were gel-eluted and cloned into T/A cloning vector and sequenced (Mention the kit and the supplier).

3.2.1.6 Restriction analysis

Restriction analysis was used to digest inserts (from vectors) and also vectors (to linearize them) for cloning and also for confirmation of the developed clones (whether the expected product size is seen on digestion). As a general rule, 1 µg DNA was used. The reaction mixture consisted of 5-10 units of enzyme, 1x final concentration of appropriate

buffer, BSA to a final concentration of 100 µg/ml and sterile water. For cloning purpose, a large volume of restriction digestion was set up (60 or 80 µl) so as to get enough digested vector and insert concentrations for efficient ligation.

3.2.1.7 Quantification of DNA

Quantification of genomic DNA, cDNA, RNA, plasmid DNA, *etc* was done by running them on 0.8% agarose gels. Approximate DNA concentration was determined by visual comparison against a known amount of standard molecular weight marker (λ DNA/50 bp ladder/100 bp ladder/ λ DNA *Eco*RI + *Hind*III double digest).

3.2.1.8 Cloning

The general procedure followed for cloning is outlined below. The details of all the clones developed in the present investigation are given in Table 3.9.

3.2.1.8.1 Preparation of vector and insert

The vectors as well as insert (to be cloned), were first isolated by setting up large volume PCR or restriction with appropriate enzymes (as mentioned earlier). The products were resolved on 0.8 % agarose gel and the desired fragments were identified on the gel using standard marker (50 bp ladder or 100 bp ladder or λ DNA *Hind*III + *Eco*RI double digest). The agarose gel slabs containing the required fragments (cut vector as well as insert) were excised out from the gel using a sharp sterile scalpel blade. DNA was eluted from the excised gel slabs using Qiagen gel elution kit as per manufacturers' instructions. For each gel slab, three volumes of QG buffer was added to 1 volume of gel and incubated at 50°C for

10 min (by vortexing intermittently). After bringing it to room temperature, one volume of isopropanol was added and the whole mix was transferred to the supplied column. After centrifugation, the flow through was discarded and the column was washed twice with PE buffer. The DNA from the column was collected by adding 25-30 μ l of elution buffer. The vector and insert DNA thus prepared were quantified by visual examination on 0.8% agarose gel.

3.2.1.8.2 Ligation

For all ligation reactions, the ratio of molar concentrations of insert to plasmid DNA used was 3:1. The amount of insert DNA needed for a known quantity of the vector DNA was calculated according to the following formula:

$$\left. \begin{array}{l} \text{Insert} \\ \text{DNA (ng)} \end{array} \right\} = \frac{(50/100 \text{ ng of vector DNA}) \times (\text{size of the insert in bp})}{\text{Size of the vector in bp}} \times 3$$

The ligation reactions consisted of 50 or 100 ng of vector DNA, insert DNA (as calculated from the formula mentioned above multiplied by 3 (for a 3:1 insert:vector ratio), 1x final concentration of the buffer, 40 units of T₄ DNA ligase and sterile water and the reaction mixture was incubated at 22°C for 5 hrs.

3.2.1.8.3 Preparation of *E.coli* competent cells

The competent cells were prepared following the protocol as given by Ausubel *et al.* (1999). *E. coli* strain DH5 α was grown at 37°C in liquid LB medium (Himedia) overnight. An aliquot of the culture was transferred to 200 ml LB medium (Himedia) and grown to an OD₅₉₀ of 0.375. The culture was distributed into four 50 ml pre-chilled sterile polypropylene

tubes and left on ice for 5-10 minutes. The cells were pelleted by centrifugation at 1600 g for 7 min (4°C). Each pellet was resuspended in 10 ml ice-cold CaCl₂ solution (15 % glycerol, 0.2 M CaCl₂, 0.1 M PIPES) and spun for 5 minutes at 1100 g (4°C). The pellet was resuspended in 10 ml ice-cold CaCl₂ solution and left on ice for 30 minutes and spun at 1100g for 5 minutes (4°C). The resultant pellet was completely resuspended in 2 ml of ice-cold CaCl₂ solution and 100 µl was aliquoted into pre-chilled sterile 1.75 ml eppendorf tubes and immediately frozen at -80°C.

3.2.1.8.4 Transformation *E. coli* competent cells

Heat shock method given by Ausubel *et al.* (1999) was followed for the transformation of competent cells with the plasmid DNA or ligation mix (which was incubated at 22°C for a minimum of 5 h). Frozen *E. coli* competent cells were thawed on ice, to which 1 ng of plasmid DNA (for control reaction) or 100 ng of ligation mix was added. The suspension was carefully mixed with pipette tip and incubated on ice for 15 min followed by heat shock at 42°C for 2 min. Cells were incubated on ice for another 2 min, 1 ml LB medium was added and incubated at 37°C for 1 h with shaking. Aliquots of the suspension were spread evenly on LA medium supplemented with appropriate antibiotic (ampicillin or kanamycin). The plates were incubated at 37°C overnight (14-16 h). The following day single colonies were picked up and inoculated in 3 ml LB medium for plasmid isolation.

3.2.1.8.5 Confirmation of cloning

The cloning at each step was confirmed by colony PCR and restriction analysis or/and PCR. The plasmid DNA of the new clones were isolated and used for this purpose.

When PCR was used for confirmation of cloning, the procedure was same as mentioned in section 3.2.3 with the use of specific primers so as to confirm the cloning and the orientation of the insert. During restriction analysis for confirmation of cloning, suitable restriction enzymes were used so as to confirm the presence of the insert as well as its orientation in the vector background. The procedure was followed as mentioned in section 3.2.3. Colony PCR was also done for a preliminary confirmation of the clones even before their plasmid DNA was isolated. For this, instead of plasmid DNA, the bacterial cells as such were used in the PCR reaction mixture. Also, the PCR reaction mixture with the bacterial cells was first heated at 95°C for 5 min. The cycling parameters were the same as that for the respective gene fragments but the annealing temperature and extension time varied according to the primers used and the size of the amplicons expected.

3.2.1.9 Development of constructs for induction of male sterility

A brief outline of the steps involved in the development of pBin-TCNN-Bar and pBin-TCAN-Bar is given here. The details and results of each of the clonings are provided in section 4.3.1 and the schematic representation is given in Figure 3.9.

nad3 and *atp9* genes were amplified from safflower and cloned into T/A cloning vector. These genomic clones were sequenced and analyzed to make sure that any PCR induced mutations are not included in them. Clones with perfect sequences (no mutations) were used further. *nad3* and *atp9* genes from these were cloned into the pCAMBIA1305.2-TCNN vector using *KpnI* and *SacI* enzymes to generate pCAMBIA1305.2-TCNN and pCAMBIA1305.2-TCAN (by replacing *orfH522* with these two genes).

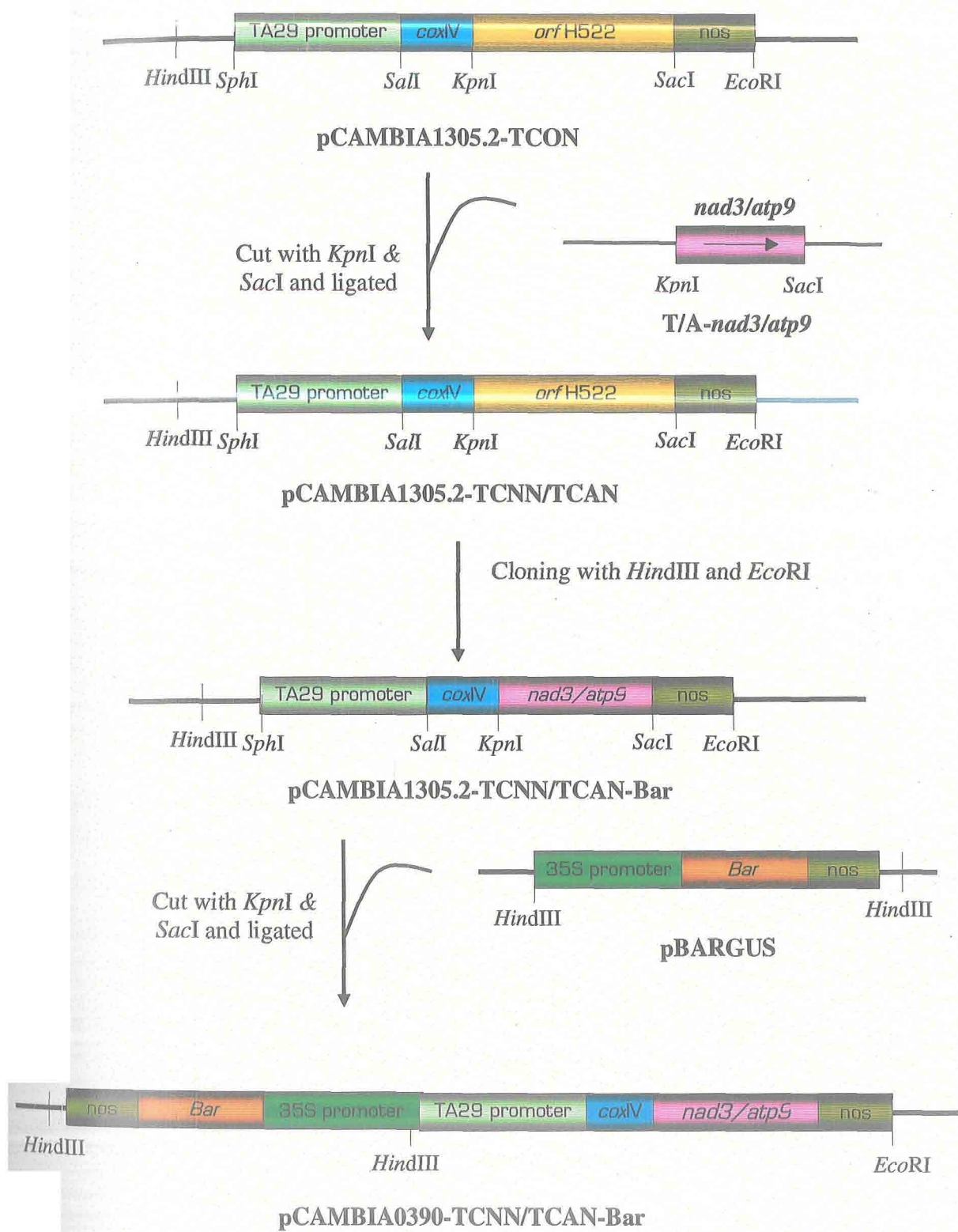


Fig. 3.9 Schematic representation of development of TCNN-Bar and TCAN-Bar constructs in pCAMBIA0390. Cloning enzymes at each step are indicated in bold.

The entire cassettes of TCNN and TCAN were then cut out using *HindIII* and cloned into pCAMBIA0390 to obtain pCAMBIA0390-TCNN and pCAMBIA0390-TCAN. The lack of any plant selection marker in this binary vector facilitated the cloning of bar gene construct into it. The pBARGUS vector was cut using *HindIII* and the entire bar gene cassette was separated out and ligated into pCAMBIA0390-TCNN and pCAMBIA0390-TCAN, which resulted in the pBin-TCNN-Bar and pBin-TCAN-Bar vectors respectively (Figures 3.9 and 3.10). These two were the vectors that were mobilized into *Agrobacterium* and used for plant transformation to develop transgenic male sterile plants.

3.2.1.10 Development of constructs for restoration of fertility

A brief outline of the steps involved in the development of fertility restoration constructs is given here. The detail of the results of each of the clonings is provided in section 4.3.2.

3.2.1.10.1 Development of full-length antisense constructs

For development of the full length antisense constructs, the pRT100 vector (which has the 35S promoter and the polyA terminator) was used. The *nad3* and *atp9* genes from the T/A-*nad3* and T/A-*atp9* clones were cloned into the pRT100 vector to obtain p-SANP and p-SAAP clones. The use of *KpnI* and *SacI* as cloning enzymes ensured the antisense orientation of the genes in these constructs (Figure 3.11). The entire SANP and SAAP cassettes were then cloned into the binary vector pCAMBIA1300 using *HindIII* enzyme and pBin-SANP and pBin-SAAP clones were obtained.

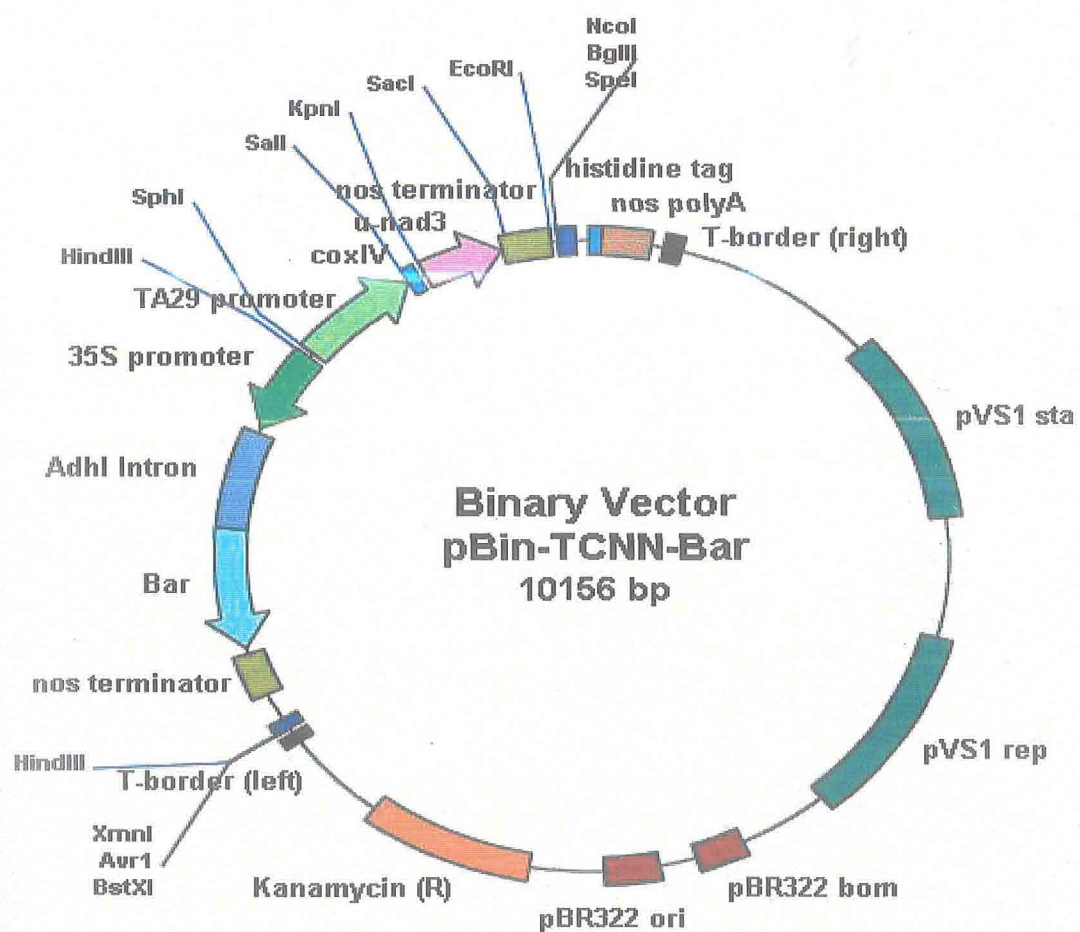


Figure 3.10. Plasmid map of the developed pBin-TCNN-Bar

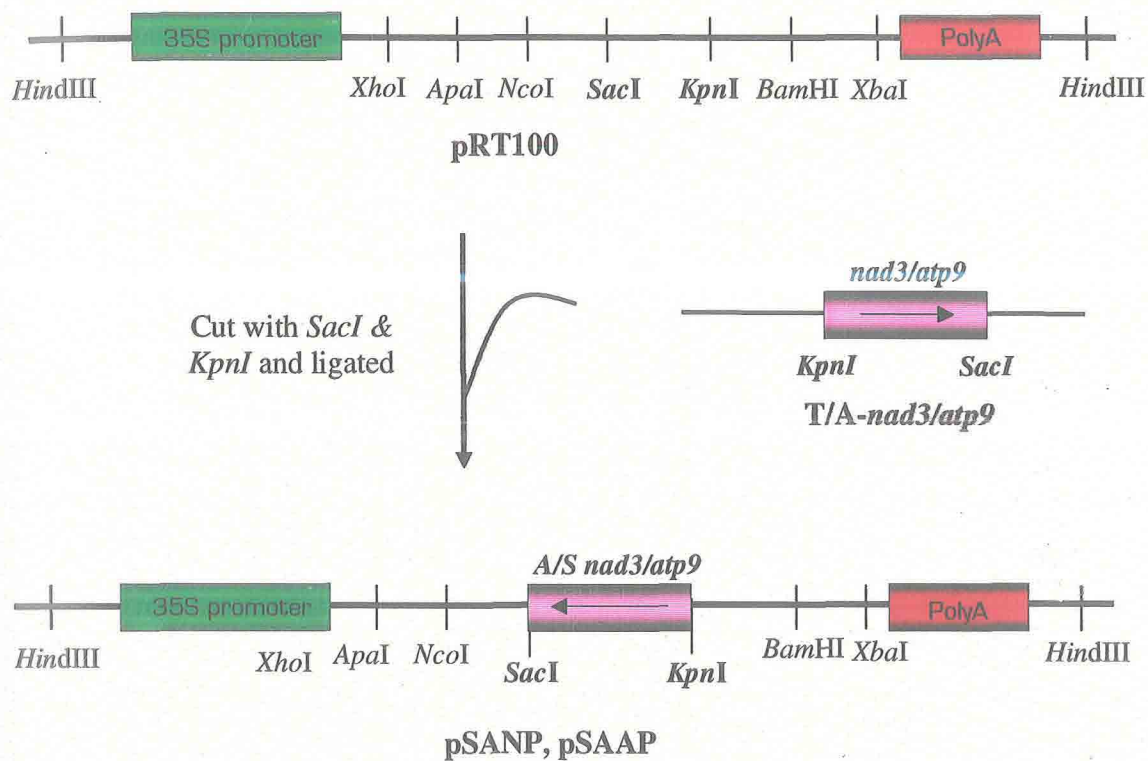


Fig. 3.11 Schematic representation of development of full-length antisense *nad3* and *atp9* constructs in pRT100. Cloning enzymes at each step are indicated in bold. The constructs were cloned into pCAMBIA1300 to develop pBin-Antisense *nad3* and pBin-Antisense *atp9* plant transformation vectors.

To develop these constructs under TA29 promoter, the promoter from T/A-TA29 was cloned into p-SANP and p-SAAP by replacing the 35S promoter and the p-TANP and p-TAAP clones were obtained (Figure 3.13). As done earlier, the entire TANP and TAAP cassettes were then cloned into the binary vector pCAMBIA1300 using *HindIII* enzyme and the pBin-TANP and pBin-TAAP clones were obtained.

3.2.1.10.2 Development of ihp-RNA constructs

For development of the ihp-RNA constructs, a new set of T/A *nad3*(ihp) and T/A *atp9*(ihp) clones were developed in such a way that these genes were flanked by *KpnI* and *SacI* sites on the 5' end and by *BamHI* and *XhoI* sites on the 3' end. The pRT100-Cat Int vector was used as the background vector. Initially the genes were isolated from T/A clones and cloned into pRT100-Cat Int using *SacI* and *XhoI* enzymes to obtain pRT100-Cat Int-antisense *nad3* and pRT100-Cat Int-antisense *atp9* clones. Into these clones, the *nad3* and *atp9* genes were cloned using *KpnI* and *BamHI* enzymes to generate pRT100-ihp *nad3* and pRT100-ihp *atp9* clones (Figure 3.12). The entire cassettes i.e., CaMV 35S promoter-ihp *nad3*-polyA terminator and CaMV 35S promoter-ihp *atp9*-polyA terminator, were excised out using *HindIII* enzyme, were cloned into *HindIII* digested pCAMBIA1300 and pBin-ihp *nad3* and pBin-ihp *atp9* clones were obtained.

For development of ihp constructs under TA29, initially TA29 from T/A TA29 was cloned into pRT100 vector using *HincII* and *XhoI* enzymes that replaced 35S promoter with TA29 to obtain p-TA29. Into this clone, the entire ihp *nad3* (940 bp) and ihp *atp9* (742 bp) (taken out from p-ihp *nad3* and p-ihp *atp9* with *XhoI*) were cloned using the *XhoI* enzyme so that p-T-ihp *nad3* and p-T-ihp *atp9* were obtained (Figure 3.14). From these vectors, the T-

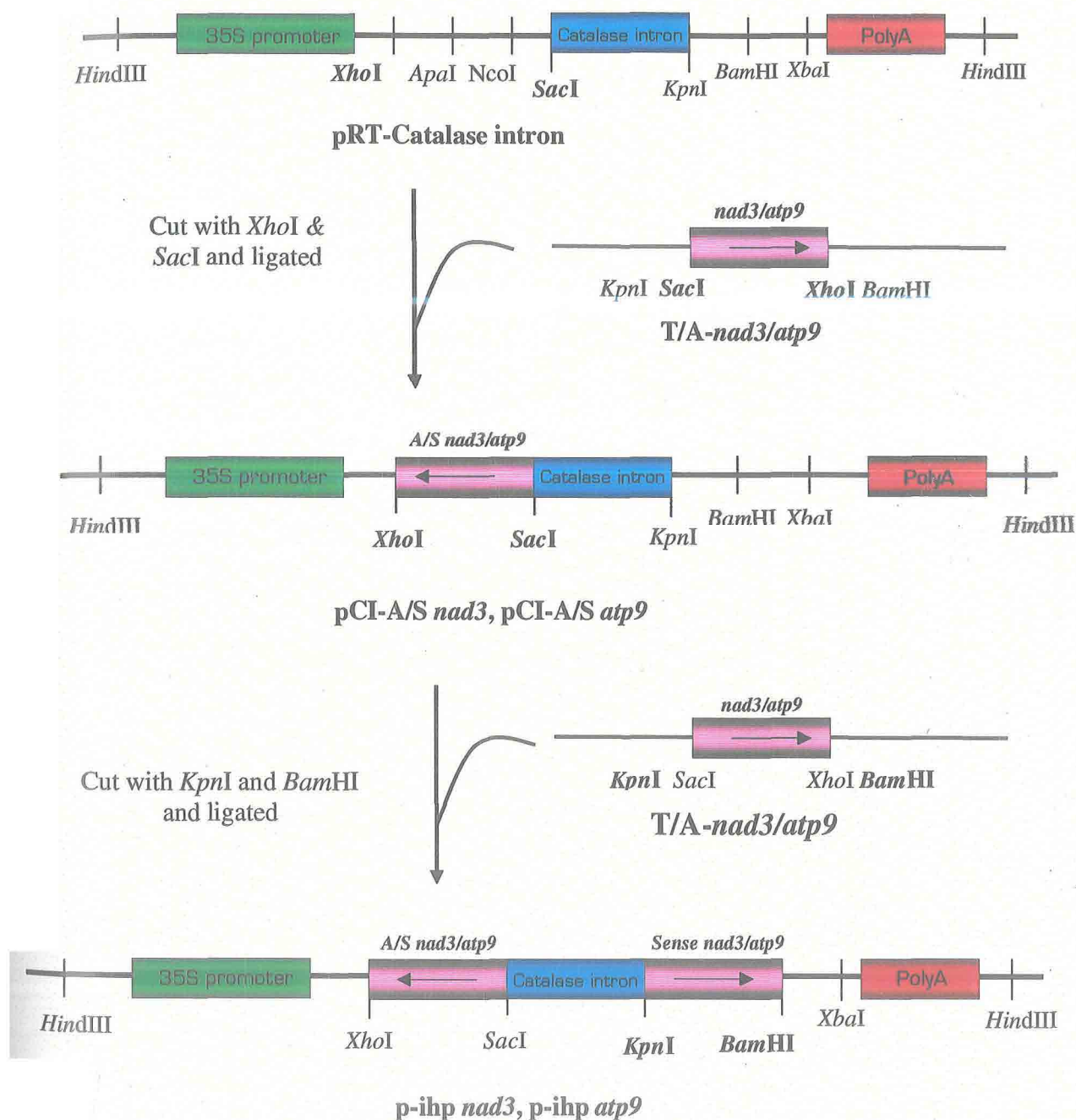


Fig. 3.12 Schematic representation of development of *ihp nad3* and *ihp atp9* constructs in pRT100. Cloning enzymes at each step are indicated in bold. The constructs were cloned into pCambia1300 to develop pBin-*ihp nad3* and pBin-*ihp atp9* plant transformation vectors.

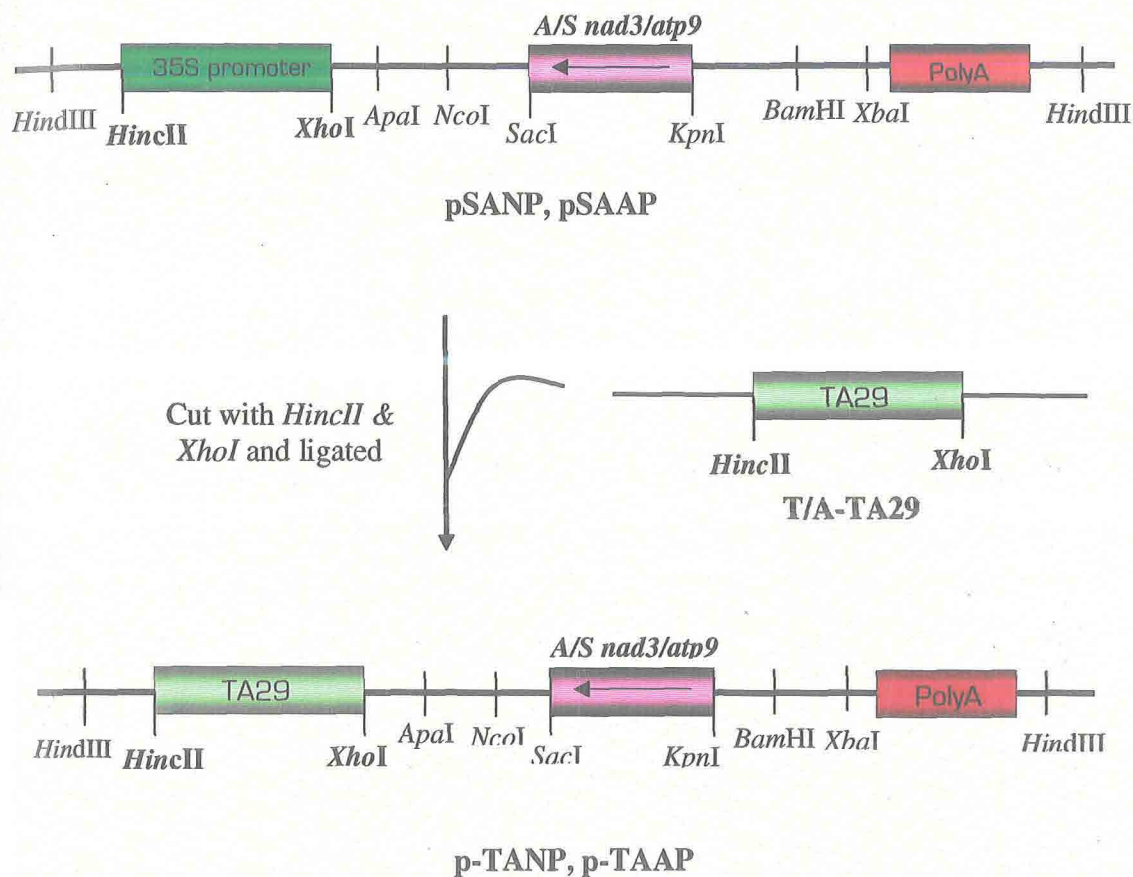


Fig. 3.13 Schematic representation of development of TA29-full length antisense *nad3* and *atp9* constructs in pRT100. Cloning enzymes at each step are indicated in bold. The constructs were cloned into pCAMBIA1300 to develop pBin-T-Antisense *nad3* and pBin-T-Antisense *atp9* plant transformation vectors.

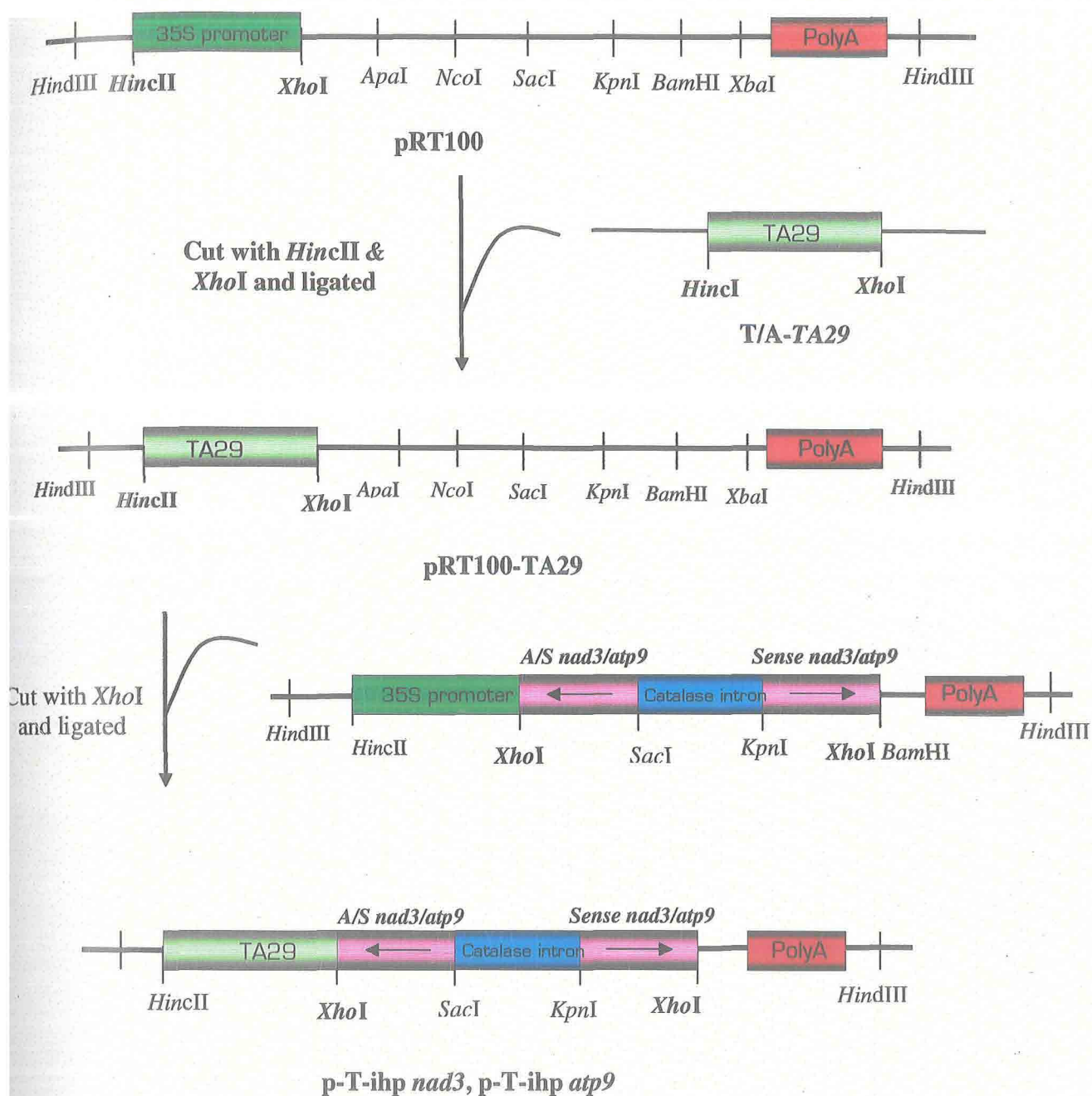


Fig. 3.14 Schematic representation of development of T-ihp *nad3* and T-ihp *atp9* constructs in **pRT100**. Cloning enzymes at each step are indicated in bold. The constructs were cloned into pCAMBIA1300 to develop **pBin-T-ihp nad3** and **pBin-T-ihp atp9** plant transformation vectors.

ihp *nad3* and T-ihp *atp9* cassettes were cloned into pCAMBIA1300 using *Pst*I enzyme to obtain pBin-T-ihp *nad3* and pBin-T-ihp *atp9* clones. All these clones were thoroughly confirmed for the presence and orientation of inserts and the results are described in the section 4.3.2.

3.2.1.11 Mobilization of the recombinant binary vectors into *Agrobacterium tumefaciens*

All the constructs developed (as mentioned above) were mobilized into the *Agrobacterium* strain LBA4404 and further confirmed before they were used for plant transformation. The general procedures followed are explained below.

3.2.1.11.1 Preparation of LBA4404 competent cells

Saturated culture of *Agrobacterium* strain LBA4404 was grown overnight. One ml of this overnight grown culture was added to 100 ml of TB medium without any antibiotics and allowed to grow unto an OD of 0.4-0.6 at A₆₀₀. The cells were pelleted down at 3000 rpm for 10 min at 4°C in a sterile falcon tube. The cells were sequentially washed in 100 ml of 0.1 M HEPES, 50 ml of 0.1 M HEPES and 50 ml of 10% glycerol by resuspension and centrifugation. The resultant pellet was resuspended in 3 ml of 10% glycerol for every 100 ml of original culture. 100 µl of aliquots were frozen and stored at -80°C.

3.2.1.11.2 Electroporation of binary vectors into *Agrobacterium tumefaciens*

Four of the binary vectors with recombinant DNA (pBin-TCNN-Bar, pBin-TCAN-Bar, pBin-T-ihp *nad3*, pBin-T-ihp *atp9*) were used to transform *Agrobacterium* strain LBA4404 by electroporation method.

The frozen *Agrobacterium* competent cells were thawed on ice and 200 ng of the recombinant binary plasmid DNA was added to competent cell mixture and mixed well. The entire mix was transferred to the center portion of prechilled cuvette (0.2 cm or 2 mm) kept on ice. This was subjected to a short pulse (5 msec) of 12.5 V per cm or 2.5 V per 2 mm, 25 μ F capacitance and 201 Ω (Ohm) resistance in an electroporator (Biorad). Immediately 1 ml of LB was added and kept back on ice. This culture was kept for shaking at 28°C for 1 h. The cells were pelleted by giving a brief spin, the supernatant was decanted and the pellet was resuspended in the left out medium ~100 μ l and spread on TA plates supplemented with kanamycin, streptomycin and rifampicin. After 2-3 days, the colonies were inoculated in 5-10 ml of TB by adding appropriate antibiotics and plasmid DNA were isolated.

3.2.1.11.3 *Agrobacterium* plasmid DNA isolation

Plasmid DNA of LBA4404 strains harboring binary recombinant plasmids were isolated from a starter culture of 10 ml each grown on rifampicin, streptomycin and kanamycin antibiotics. Fully-grown culture was incubated on ice for 5-10 min and the cells were pelleted at 3000 rpm for 15 min. The pellet was resuspended in 500 μ l of GET (50 mM glucose, 25 mM Tris-Cl pH 5.8, 10 mM EDTA) and 250 μ l lysozyme (@ 4 mg/ml) and incubated on ice for 10-15 min. 1 ml of NSW (0.2 N NaOH, 1% SDS) was added and incubated at room temperature for 10 min. To the same tube, 750 μ l of 3 M potassium acetate (pH 5.2) was added and incubated on ice for 15 min. Centrifugation was done at 15000 rpm for 15 min at 4°C. To the supernatant, equal volume of phenol: chloroform: isoamylalcohol (25:24:1) was added and mixed well. To the aqueous phase, equal volume of chloroform: isoamylalcohol (24:1) was added, mixed well and spun at 15000 rpm for 10

min. The aqueous phase containing plasmid was precipitated with double volume of ethanol and kept at -20°C for 10 min. The pellet was washed with 70% ethanol, air-dried and dissolved in 50 μl of T_{10} .

3.2.1.11.4 Confirmation of constructs in *Agrobacterium*

3.2.1.11.4.1 3-Ketoglycoside test for confirmation of *Agrobacterium* strain

This test was performed to check whether the strain was *Agrobacterium* or any other contaminant. *Agrobacterium* species grown on lactose make an enzyme called hexa pyranoside cytochrome C oxido-reductase that converts lactose to 3-keto-lactose, which in turn converts cupric oxide of Benedict's solution to cuprous oxide, a yellow colored precipitate. *Agrobacterium* strains (with recombinant DNA) streaked on 3-ketolactose medium (1% lactose, 0.1% yeast extract, 2% bacto-agar) were flooded with Benedicts reagent (17.3% sodium citrate, 10% sodium carbonate anhydrous, 1.74% copper sulphate). Yellow hallow of Cu_2O appears wherever 3-ketolactose is produced by *Agrobacterium*. By using this test all the *Agrobacterium* cultures were confirmed as *Agrobacterium* strains.

3.2.1.11.4.2 Confirmation by restriction endonuclease digestion or PCR

The constructs were finally confirmed at *Agrobacterium* level also since recombinations are known to take place during transformation of *Agrobacterium*. Whenever the plasmid DNA was of good quality (as ascertained by viewing on agarose gels) as well as good quantity (at least 200 $\text{ng}/\mu\text{l}$), restriction digestion was used for confirmation. The reaction mixture consisted of 0.8 to 1.0 μg of plasmid DNA, 10-20 units of restriction

enzyme, 1x final concentration of appropriate buffer, BSA to a final concentration of 100 µg/ml and milliQ water to make up the final volume. When plasmid DNA concentration was very low (less than 200 ng/µl), PCR (as per the procedure mentioned above in section 3.2.3) was used for confirmation of the clones.

3.2.1.11.4.3 Confirmation by reverse mating

For further confirmation of orientation of cloning of the required gene cassette in binary vector, reverse mating (transfer of the binary plasmid back into *E. coli*) was done. Chemically competent *E. coli* cells of DH5α were transformed with ~50 ng of *Agrobacterium* plasmid DNA using the standard heat shock method described earlier. The plasmid DNA from the *E. coli* was subjected to appropriate restriction analysis or PCR.

3.2.2 Tissue culture and Transformation

3.2.2.1 Immature embryo culture in safflower

3.2.2.1.1 Collection of developing seeds

Young safflower capitula that have yellow or orange florets were collected from the field. Red or even darker florets indicate post bloom stage and are not desirable. Other parts of the capitula like spiny sepals, florets etc were discarded and the young achenes (seeds) alone were collected.

3.2.2.1.2 Isolation of immature embryos

The seeds were surface sterilized with 0.1% mercuric chloride for 10 minutes and then rinsed four times with sterile water. In the water used for last wash, a pinch of

streptomycin sulphate was added. The seeds were then blotted dry and the developing embryos were excised out carefully by using sterile forceps and blades. All these operations were performed under aseptic conditions.

3.2.2.1.3 Culture of immature embryos

The embryos isolated were cultured on different media combinations (primary media) to determine the media combination, which is most effective for somatic embryogenesis (Table 3.10). The responding explants were then transferred to five secondary media combinations and the most effective primary – secondary media combinations were deduced. Tables 3.10 and 3.11 give the different primary and secondary media combinations tried. An experiment was also done to determine the size of immature embryos that are optimum for culture. The embryos which occupied less than half space inside the seed (as seen while isolation) and those occupying half the seed were designated as 'small' and 'medium' sized embryos respectively. The embryos that were big enough to occupy whole of the seed were designated as 'large' embryos.

3.2.2.2 Transformation of Safflower and Tobacco

3.2.2.2.1 Media preparation

Ready made mix of Murashige and Skoog medium supplemented with different hormones and 0.8% agar as gelling agent was used. The required media at various stages of explant growth in tissue culture work are as listed in the Tables 3.12 and 3.13.

Antibiotics and hormone stocks were filter sterilized and stored at -20°C. All media with required hormones were autoclaved for 20 min at 121°C, 103.5 kPa pressure and stored at

Table 3.10 Different primary media combinations tested for immature embryo culture

Designation	Media combination
E ₁	MS + 3% sucrose + TDZ 0.5 mg/l + NAA 1.0 mg/l
E ₂	MS + 10% sucrose + TDZ 0.5 mg/l + NAA 1.0 mg/l
E ₃	MS + 3% sucrose + TDZ 0.5 mg/l
E ₄	MS + 10% sucrose + TDZ 0.5 mg/l
E ₅	MS + 3% sucrose + TDZ 1.0 mg/l
E ₆	MS + 10% sucrose + TDZ 1.0 mg/l
E ₇	MS + 3% sucrose + 2,4-D 0.05 mg/l
E ₈	MS + 3% sucrose + 2,4-D 0.2 mg/l
E ₉	MS + 3% sucrose + 2,4-D 0.5 mg/l
E ₁₀	MS + 6% sucrose + 2,4-D 0.05 mg/l
E ₁₁	MS + 6% sucrose + 2,4-D 0.2 mg/l
E ₁₂	MS + 6% sucrose + 2,4-D 0.5 mg/l
E ₁₃	MS + 9% sucrose + 2,4-D 0.05 mg/l
E ₁₄	MS + 9% sucrose + 2,4-D 0.2 mg/l
E ₁₅	MS + 9% sucrose + 2,4-D 0.5 mg/l
E ₁₆	MS + 12% sucrose + 2,4-D 0.05 mg/l
E ₁₇	MS + 12% sucrose + 2,4-D 0.2 mg/l
E ₁₈	MS + 12% sucrose + 2,4-D 0.5 mg/l

Table 3.11 Different secondary media combinations tested for immature embryo culture

Designation	Media combination
S ₁	MS + BAP 0.2 mg/l
S ₂	MS + kinetin 0.2 mg/l
S ₃	MS + kinetin 0.5 mg/l
S ₄	MS + TDZ 0.5 mg/l + NAA 0.1 mg/l
S ₅	MS + TDZ 0.5 mg/l + NAA 0.5 mg/l

Table 3.12 Different media combinations used during tissue culture

Medium	Composition
Basal medium	MS powder + 1x $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$
Half-strength MS	Half-strength MS
Co-cultivation medium (for safflower)	SV ₁₇ (MS powder + 1x CaCl_2 + NAA 0.2 mg/l + TDZ 0.2 mg/l)
Shoot induction medium (for safflower)	SV ₁₇ (MS powder + 1x CaCl_2 + NAA 0.2 mg/l + TDZ 0.2 mg/l)
Shoot multiplication medium (for safflower)	MS powder + 1x CaCl_2 + BA 0.5 mg/l or BA 0.2 mg/l
Shoot elongation medium (for safflower)	MS powder + 1x CaCl_2 + BA 0.5 mg/l or BA 0.2 mg/l or kinetin 0.5 mg/l
Rooting medium (for safflower)	RM ₁ (Half MS + IBA 2.0 mg/l + phloroglucinol 2.0 mg/l) or RM ₂ (Half MS + NAA 0.5 mg/l)
Co-cultivation medium (for tobacco)	BN (MS powder + 1x CaCl_2 + BA 1.0 + NAA 0.1 mg/l)
Shoot induction medium (for tobacco)	BN (MS powder + 1x CaCl_2 + BA 1.0 + NAA 0.1 mg/l)
Shoot multiplication medium (for tobacco)	MS powder + 1x CaCl_2 + BA 0.5 mg/l
Rooting and elongation medium (for tobacco)	Half-strength MS

Table 3.13 Media along with the antibiotics/herbicides used for selection of transformants at different stages

Constructs	Selection I	Selection II	Selection III	Rooting
Safflower				
TCNN-Bar	SV ₁₇ + phosphinothricin	Shoot multiplication medium + phosphinothricin 1.0 mg/l + cefatoxime 250 mg/l	Shoot elongation medium + phosphinothricin 1.0 mg/l + cefatoxime 250 mg/l	RM ₁ or RM ₂ + cefatoxime 250 mg/l
TCAN-Bar	0.5 mg/l + cefatoxime 250 mg/l			
T ihp <i>nad3</i>	SV ₁₇ + hygromycin 10 mg/l + cefatoxime 250 mg/l	Shoot multiplication medium + hygromycin 15 mg/l + cefatoxime 250 mg/l	Shoot elongation medium + hygromycin 20 mg/l + cefatoxime 250 mg/l	RM ₁ or RM ₂ + cefatoxime 250 mg/l
T ihp <i>atp9</i>				
Tobacco				
TCNN-Bar	BN + phosphinothricin 3.0 mg/l + cefatoxime 250 mg/l	BN + phosphinothricin 3.0 mg/l + cefatoxime 250 mg/l	Shoot multiplication medium + phosphinothricin 1.0 mg/l + cefatoxime 250 mg/l	Half strength MS + cefatoxime 250 mg/l
TCAN-Bar				
T ihp <i>nad3</i>	BN + hygromycin 30 mg/l + cefatoxime 250 mg/l	BN + hygromycin 30 mg/l + cefatoxime 250 mg/l	Shoot multiplication medium + hygromycin 20 mg/l + cefatoxime 250 mg/l	Half strength MS + cefatoxime 250 mg/l
T ihp <i>atp9</i>				

25°C. Prior to use, required antibiotics were added to melted and cooled media and poured into suitable glassware.

3.2.2.2.2 Surface sterilization of seeds for obtaining aseptic seedlings

Seed treatment is a must for obtaining healthy aseptic seedlings for further downstream tissue culture work. Good, bold and healthy safflower seeds were selected and washed under running water for 2-3 h after adding 2-3 drops of Labolene (detergent). Then the seeds were surface sterilized with 0.1% HgCl₂ (mercuric chloride) for 12-15 min followed by 5 min washing with sterile distilled water four times with continuous shaking. During the fourth wash, a pinch of streptomycin sulphate, a generic antibiotic was added. Seeds were dried on sterile blotting paper and inoculated on half strength MS media tubes at 3-4 seeds per tube.

This step was not needed for tobacco as shoots, that were continuously being maintained on MS + BA 0.5 mg/l or ½ MS in the biotechnology laboratory, were used for co-cultivation.

3.2.2.2.3 Co-cultivation

8-12 day-old aseptic safflower seedlings were used for co-cultivation. The *Agrobacterium* culture (strain LBA4404) having the desirable recombinant binary vector was grown overnight in YEP or LB liquid medium with streptomycin (25 mg/l), kanamycin (50 mg/l) and rifampicin (25 mg/l). The following day, hypocotyls, cotyledonary leaves and roots were cut to 0.5 cm size under laminar air flow and kept for 10 min shaking (180 rpm) separately in sterile tubes containing the *Agrobacterium* culture diluted to 0.1 OD with liquid MS. After subjecting these explants to vacuum infiltration for half-an-hour they were

dried on sterile blotting paper for few minutes and placed on shoot induction medium without selection pressure. The plates were incubated in dark for 48 hrs at 23-25°C.

In case of tobacco, leaves from shoots that were being maintained in the lab were used. The leaves were cut into 0.5 × 0.5 cm sized pieces and incubated in *Agrobacterium* culture diluted to 0.1 OD with liquid MS for 5 minutes with gentle shaking. They were then blot dried and inoculated onto BN medium (without any selection agent). The plates were incubated in dark for 48 hr at 23-25°C as for safflower.

3.2.2.2.4 Selection and plant regeneration

After 48 h of incubation, the safflower explants were transferred to shoot induction medium (Selection I) having phosphinothricin (when constructs for induction of male sterility were used) or hygromycin (when constructs for restoration of fertility were used) and cefotaxime and incubated for 15 days. Explants, which appeared healthy and greenish, were transferred to shoot multiplication medium (Selection II) having phosphinothricin or hygromycin (as per the construct being used) and cefotaxime and were incubated for 15 days. Explants, which showed shoot formation, were transferred to (shoot elongation) selection III medium. The compositions of media used during different selections are given in Tables 3.12 and 3.13. When shoots were large enough, they were transferred to BA (0.5 mg/l) medium in flasks with no selection pressure but with cefatoxime (250 mg/l). At each cycle of selection the dried and darkened explants (untransformed) were discarded.

For tobacco, the explants after co-cultivation were transferred to BN medium with selection agent for two cycles of 15 days each, after which the explants that responded were transferred to medium containing BA 0.5 mg/l with selection agent. The concentrations of

antibiotics/herbicides used at each stage of selection and for different explants are also mentioned in Table 3.13.

Transformation efficiency, for both tobacco and safflower, was calculated by taking in to account the number of explants used for co-cultivation and number of the explants that gave rise to shoots at the end of third selection. It was calculated using the following formula

$$\text{Transformation efficiency} = \frac{\text{No. of explants which gave rise to shoots after third selection}}{\text{No. of explants used for co-cultivation}}$$

3.2.2.2.5 Shoot multiplication, elongation and rooting

Well established and healthy safflower shoots were multiplied on MS with BA (0.5 mg/l) medium having cefatoxime (250 mg/l). Individual shoots were kept on elongation medium with cefotaxime (250 mg/l). Elongated shoots showing 2-4 nodes were transferred to rooting medium RM₁ or RM₂ containing cefotaxime (250 mg/l).

In case of tobacco, regenerated shoots were maintained on MS with BA (0.5 mg/l) medium having cefatoxime (250 mg/l). Individual shoots were then transferred to growth regulator free ½ MS medium having cefatoxime (250 mg/l) on which the shoots elongated as well as formed roots.

3.2.2.2.6 Acclimatization and hardening

Rooted plants (both safflower and tobacco) were carefully removed from rooting medium and the roots were washed carefully under running water (to remove the agar). They were then carefully transplanted into sterile vermiculite in small plastic plots and

wetted thoroughly with 1/4th strength Hoagland's solution and covered completely with plastic covers to maintain high humidity. After 5-7 days, the plastic covers were cut at the edges to permit aeration. After another 5-7 days, the covers were removed from the pots. After another week in the tissue culture lab, the small pots with plants were transferred to the green house. After 7-10 days, the plants were transplanted into larger sized pots having a mixture of soil and vermiculite. Throughout this period, the safflower plants were wetted with 1/2 or 1/4th strength Hoagland's solution, whereas tobacco plants grew healthy even with only water (no Hoagland's solution was used).

3.2.2.3 Confirmation of the developed transgenics

All the developed safflower and tobacco transgenics were confirmed by PCR analysis, herbicide/antibiotic sensitivity assay, morphological observations and pollen studies.

3.2.2.3.1 PCR analysis

Putative transgenics obtained with different constructs were confirmed using PCR amplification (after DNA was isolated as mentioned in 3.2.1.2.1) of specific component sequences present in the construct. The different primers used for different constructs and the expected amplicons sizes are given in Table 3.14. The PCR programme and conditions were the same as mentioned in section 3.2.3.

Table 3.14 Primers used and amplicons obtained during confirmation of transgenics by PCR analysis

Construct	Primer Combination	Expected amplicons
LBA4404: pBin-TCNN-Bar	<i>SphI</i> TA29 F + <i>KpnI</i> <i>coxIV</i> R	645 bp
	<i>SphI</i> TA29 F + <i>SacI</i> <i>nad3</i> R	1.0 kb
LBA4404: pBin-TCAN-Bar	<i>SphI</i> TA29 F + <i>KpnI</i> <i>coxIV</i> R	645 bp
	<i>SalI</i> <i>coxIV</i> F + <i>SacI</i> <i>atp9</i> R	912 bp
LBA4404: pBin-T-ihp <i>nad3</i>	<i>SphI</i> TA29 F + <i>KpnI</i> Cat Int R	1.1 kb
	<i>SstI</i> Cat Int F + <i>BamHI</i> <i>XhoI</i> <i>nad3</i> R	565 bp
LBA4404: pBin-T-ihp <i>atp9</i>	<i>SphI</i> TA29 F + <i>KpnI</i> Cat Int R	1.0 kb
	<i>SstI</i> Cat Int F + <i>BamHI</i> <i>XhoI</i> <i>atp9</i> R	465 bp

3.2.2.3.2 Phosphinothricin/Hygromycin Assay

The transgenic plants harbouring the constructs TCNN-Bar and TCAN-Bar which were transferred to the green house were analyzed for phosphinothricin sensitivity since the *bar* gene expression is expected to confer phosphinothricin resistance to these plants. 5 µl of phosphinothricin solution (1 mg/ml) was applied and gently applied on the surface of a clean leaf on each of the plants. After 5-6 days the portion of phosphinothricin application was observed and observations were recorded as sensitive or resistant reaction. Simultaneously, a control plant (non-transgenic) was also assayed in a similar manner. In a similar manner the *ihp nad3* and *ihp atp9* transgenic plants were assayed for hygromycin sensitivity (5 µl of 15 mg/ml conc.). This analysis was done for both safflower and tobacco transgenic plants.

3.2.2.3.3 Morphological observations

All the transgenic plants which were acclimatized in the green house were observed for any differences with respect to vegetative traits like plant height, number of leaves, number of flowers etc. More specifically the flowers of the male sterility transgenics were observed for any abnormalities with respect to floral organs. In those plants where seeds/capsules were set, they were observed for any size differences in comparison to the control untransformed plants.

3.2.2.3.4 Pollen Fertility Studies

The few safflower plants which survived in the green house and reached flowering stage were analyzed for pollen fertility. The florets from each plant were placed on a clean glass slide and observed under a dissection microscope. The anthers were separated

carefully and crushed (in order to spread the pollens). The sample was then stained using a drop of 0.1% acetocarmine stain, covered with a coverslip gently avoiding air bubbles. The slides were viewed under a light microscope after 3-4 hours. The pollen, which appeared densely stained, were designated as fertile pollen and those which did not take up stain were designated as sterile pollen. Similarly, the anthers from flowers of the tobacco transgenic plants, were also crushed, stained with 0.1% acetocarmine and observed for pollen fertility. The pollen from the male sterile plants were incubated in the pollen germination medium for 3-5 hrs at 25°C and viewed under microscope for normal/abnormal germination. The pollen germination medium included 5% sucrose and 3.75 ppm boric acid at pH5.8 (Zabaleta *et al.*, 1996).

CHAPTER IV

RESULTS

CHAPTER IV

RESULTS

The present investigation, carried out at the Directorate of Oilseeds Research, Hyderabad, aimed at developing transgenic male sterile and fertility restorer lines in safflower. The main hypothesis, on which this study was based, is that RNA editing of mitochondrial genes is crucial for functioning of mitochondria. Hence unedited mitochondrial genes could be employed to induce transgenic male sterility by targeting the protein encoded by unedited version of the gene into mitochondria. So, as the first step, important safflower mitochondrial gene(s) that undergo extensive editing were identified. The second step involved development of gene cassettes using the identified genes, for induction of transgenic male sterility as well as restoration of fertility. The third and fourth steps involved using these constructs to transform safflower and tobacco (as a control system) followed by confirmation of the developed transgenics. Attempt was also made to induce somatic embryogenesis in safflower under the present study. The results obtained during the course of investigation are presented here.

4.1 Identification of suitable mitochondrial genes by RNA editing studies:

4.1.1 PCR and RT-PCR for *atp6* in safflower

Based on the literature survey, it was intended to study RNA editing status of *atp6* in safflower. The basis for this was that change in editing pattern of *atp6* has been found to have a role in cytoplasmic male sterility in rice (Iwabuchi *et al.*, 1993) and sorghum (Howad and Kempken 1997; Howad *et al.*, 1998). Hence it was hypothesized that *u-atp6* gene could

induce male sterility in safflower. As a precautionary first step, the status of editing in *atp6* transcripts of safflower was to be empirically determined so that it would provide stronger basis for use of *u-atp6* as the candidate gene.

As no information regarding *atp6* sequences in safflower was available, primers (*KpnI atp6* FI and *SacI atp6* RI) were designed based on sunflower (belonging to same family *Asteraceae*) *atp6* sequence (X82387) available at NCBI. As the primers were designed based on sunflower sequences, for all the procedures sunflower was used as the positive control. Mitochondrial DNA as well as RNA was isolated from both safflower and sunflower. Their quantities as well as quality were assessed by agarose gel electrophoresis. Mitochondrial DNA showed intact high molecular weight bands indicating good quality. Mitochondrial RNA showed two intact distinct and clear bands (corresponding to 18S rRNA, 26S rRNA) with slight smear in the background, indicating a good RNA preparation (Figure 4.1).

PCR with mitochondrial DNA for *atp6* gave two faint bands of 906 bp and 250 bp in safflower and an expected 906 bp band in sunflower (Figure 4.2). However, repetition of this PCR in safflower did not yield 906 bp long band consistently, while the 250 bp band was seen consistently in safflower. In contrast, PCR with sunflower DNA consistently gave a 906 bp band. Therefore the first time amplification of 906 bp in safflower was considered artifact of PCR and it also hinted the possibility that the primer binding sequences present in sunflower, were absent/modified in safflower. RT-PCR using the mitochondrial RNA (DNase treated) of safflower, showed only a single thick consistent band of ~250 bp (and no 906 bp band at all) (Figure 4.3).

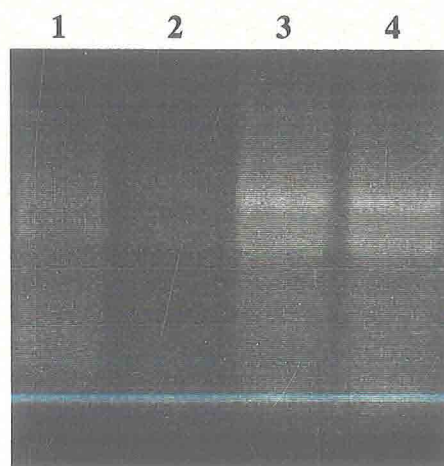


Fig. 4.1 Mitochondrial RNA of sunflower and safflower

Lanes 1 and 2. Sunflower

Lanes 3 and 4. Safflower.

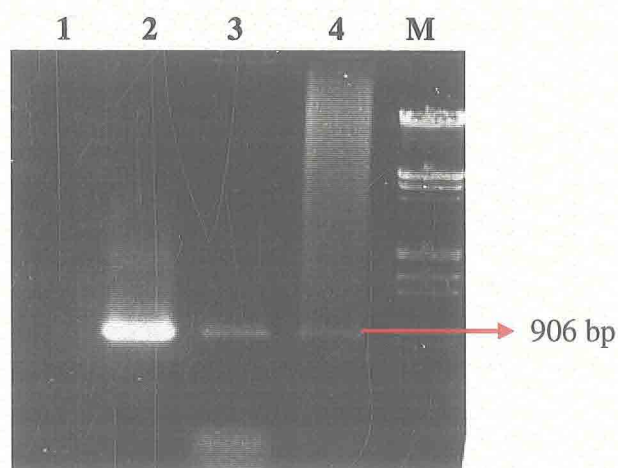


Fig. 4.2 PCR amplification of *atp6* with mitochondrial DNA of safflower and sunflower

Lane 1. Undiluted sunflower mitochondrial DNA; 2. Diluted sunflower mt DNA; 3. Undiluted safflower mt DNA; 4. Diluted safflower mt DNA; M. λ (*Hind*III + *Eco*RI) double digest marker.

4.1.2 Designing of additional primers for *atp6* gene

The above result indicated that the entire portion of *atp6* gene might not be conserved in safflower with respect to the sunflower *atp6*. Comparison of sunflower *atp6* sequence with other dicot species indicated that 1 to 150 bp at the 5' portion was not conserved among different species. So two sets of forward (*KpnI atp6* FII and *KpnI atp6* FIII) and reverse primers (*SacI atp6* RII and *SacI atp6* RIII) were designed within the conserved portion, based on the sunflower sequence (depicted in Figure 4.4).

PCR was set up with sunflower and safflower mitochondrial DNA with all possible combinations of these primers (Figures 4.5a & b). Table 4.1 gives the different primer combinations tested, expected amplicons (based on sunflower sequence) and the amplicons actually obtained in the present study. Expected amplicons were not obtained whenever the FI primer was used, clearly supporting our earlier result that *atp6* of safflower may not be conserved towards the 5' end (spanning the FI primer binding site).

To understand the status of conservation of the sequence at the RNA level, RT was done using the R1 primer so as to ascertain the size of safflower *atp6* cDNA. Using this product, RT-PCR was set up using all three forward primers (FI, FII, FIII). It was found that only FIII+RI gave a thick 250 bp band whereas with the other two (FI and FII) forward primers amplicons were not obtained (Figure 4.6; Table 4.1). These results demonstrated that *atp6* in safflower differed upstream of FIII site in comparison to sunflower gene sequence.

Table 4.1 Amplicons obtained with different primer combinations for *atp6*

	Expected amplicons	Amplicons obtained		
		Sunflower PCR	Safflower PCR	Safflower RT-PCR
FI + RI	906 bp	906 bp	906 bp*, 250 bp ^f	250 bp ^f
FI + RIII	382 bp	382 bp	-	ND
FI + RII	224 bp	224 bp	-	ND
FII + RI	733 bp	733 bp	733 bp	-
FII + RIII	210 bp	210 bp	210 bp	ND
FII + RII	51 bp	NV	NV	ND
FIII + RI	706 bp	706 bp	706 bp	706bp
FIII + RIII	182 bp	182 bp	182 bp	ND

NV-not visible on the gel due to small fragment size

*- product not obtained consistently f - very faint band obtained

ND-Not determined



Fig. 4.3 RT-PCR for *atp6* with safflower mitochondrial RNA

Lane 1 & 2. safflower RT-PCR; M. 100bp ladder
Primers FI and RI were used.

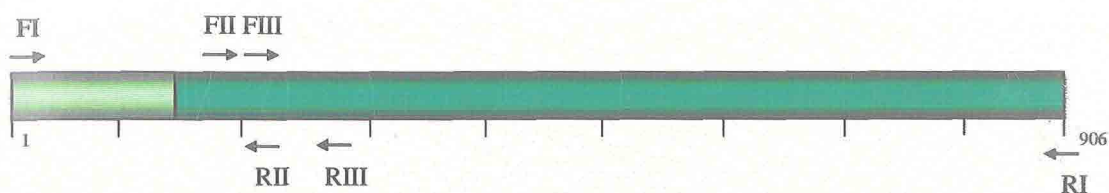


Fig. 4.4 Designing of primers for *atp6* based on sunflower *atp6* gene sequence

 Region not conserved in other species
 Region conserved in other species

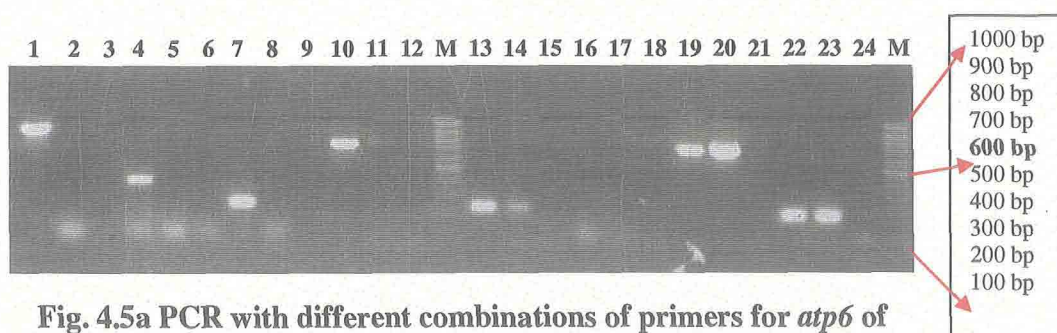


Fig. 4.5a PCR with different combinations of primers for *atp6* of sunflower and safflower

Lanes 1,2&3. FI + RI; 4,5&6. FI + RIII; 7,8&9. FI + RII; 10,11&12. FII+RI; 13,14&15 FII + RIII; 16,17&18 FII + RII; 19,20&21. FIII + RI; 22,23&24. FIII + RII; 1,4,7,10,13,16,19&22. sunflower DNA; 2,5,8,11,14,17,20&23. safflower DNA; 3,6,9,12,15,18,21&24. blanks.

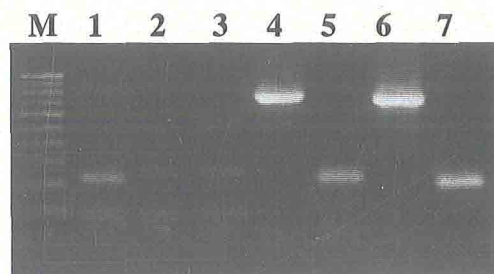


Fig. 4.5b PCR with safflower for *atp6*

Lane 1. FI + RI; 2. FI + RIII; 3. FI + RII; 4. FII + RI; 5. FII + RIII; 6. FIII + RI; FIII + RIII; M. 100bp DNA ladder.

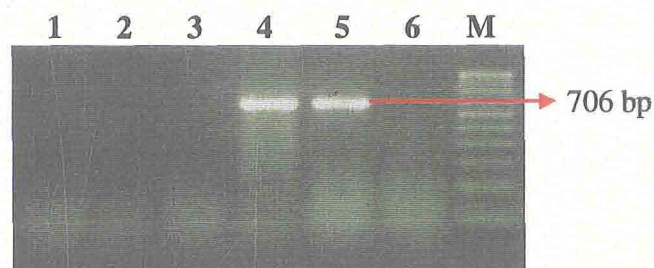


Fig. 4.6 RT-PCR with different primer combinations for *atp6* in safflower

Lane 1& 6. primers FI + RI; 2&3. primers FII + RI; 4&5. primers FII + RI; M. 100bp DNA ladder.

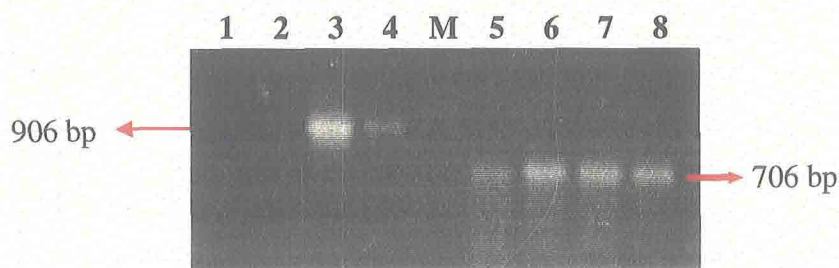


Fig. 4.7 PCR for *atp6* FIRI and FIIIRI with safflower, sunflower and castor

Lanes 1&5. safflower mt DNA; 3&7. sunflower genomic DNA; 2&6. safflower genomic DNA; 4&8. castor genomic DNA; 1,2,3&4. amplification with FI + RI; 5,6,7&8. amplification with FIII + RI; M. 100bp DNA ladder.

4.1.3 Cloning of Safflower *atp6* FIIIRI and sequence analysis

To compare the conserved region of *atp6* gene between safflower and sunflower, the FIIIRI product (706 bp) was amplified from safflower mitochondrial DNA along with the FIRI (906 bp) product from sunflower mitochondrial DNA (Figure 4.7). The bands were cloned into T/A cloning vector. The presence of the insert was confirmed by colony PCR (Figure 4.8 and 4.9). The restriction analysis with *SacI* enzyme indicated the orientation of the insert. Presence of a 731 bp band indicated antisense and no visible insert band indicated sense orientation of the insert (Figure 4.10). One confirmed positive clone each of sunflower (FIRI) and safflower (FIIIRI) were sequenced (Microsynth company) and the sequences were compared. It was found that the 706 bp of safflower corresponded exactly with the same portion of sunflower. This confirmed our earlier hunch that the non-conservation was at the 5' end of the *atp6* gene.

4.1.4 5' Rapid Amplification of cDNA Ends (5' RACE) and cloning the product

To obtain the full length genomic and cDNA clones of safflower, the 5' sequence of the *atp6* gene had to be determined. 5' RACE (Rapid Amplification of cDNA Ends) that is usually employed to determine the 5' end of the transcript region was attempted.

The safflower mitochondrial RNA was used and an RT reaction using the RIII primer (which corresponded to a region well within the conserved 5' region of *atp6*) was set up. PolyC oligonucleotide was ligated to the cDNA using RNA ligase and the resultant product was used for 5' RACE. PolyG and RIII primers were used for PCR and amplicon of around 500 bp was obtained (Figure 4.11), which was gel-eluted and cloned into T/A vector. The presence of the insert was confirmed by PCR (Figure 4.11) as well as restriction

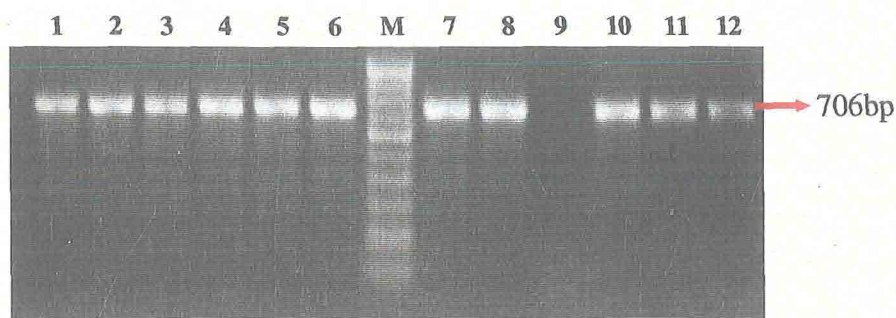


Fig. 4.8 Colony PCR for safflower T/A-*atp6* FIIIRI clones

Lanes 1 to 12. Clones 1 to 12 of T/A safflower FIIIRI; Clone 9 is negative (no insert); M. 100bp DNA ladder.

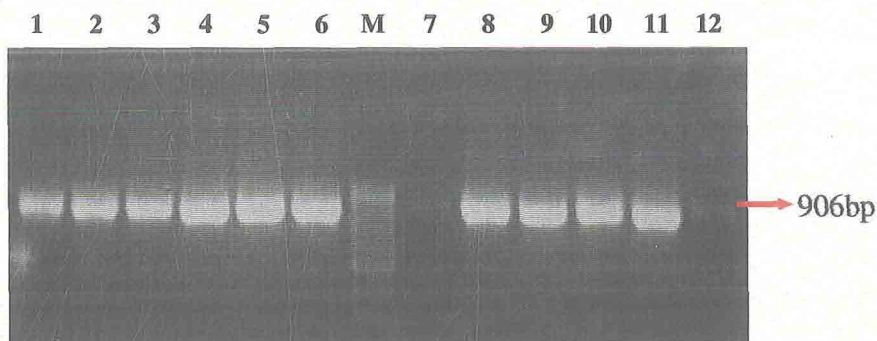


Fig. 4.9 Colony PCR for sunflower T/A-*atp6* FIRI clones

Lanes 1 to 12. Clones 1 to 12 of T/A sunflower FIRI; Clone 7&12 showed very faint amplification; M. 100bp DNA ladder.

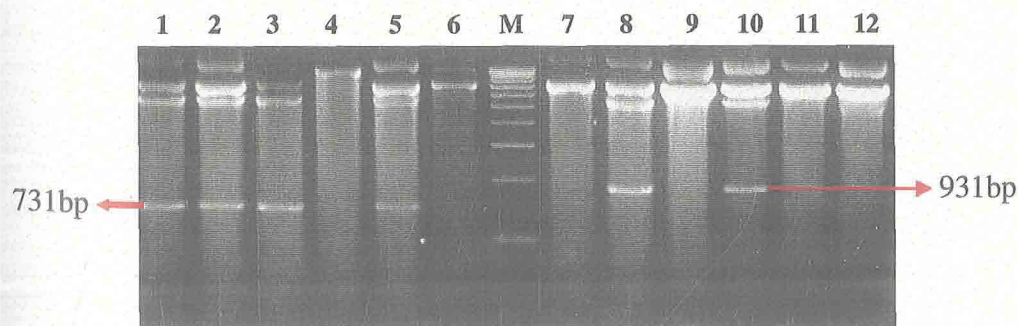


Fig. 4.10 Restriction of safflower T/A-*atp6* FIIIRI and sunflower T/A-*atp6* FIRI with *Sac*I to check the orientation

Lanes 1 to 6. T/A safflower FIIIRI clones; 7 to 12. T/A sunflower FIRI clones; M. 500 bp ladder.

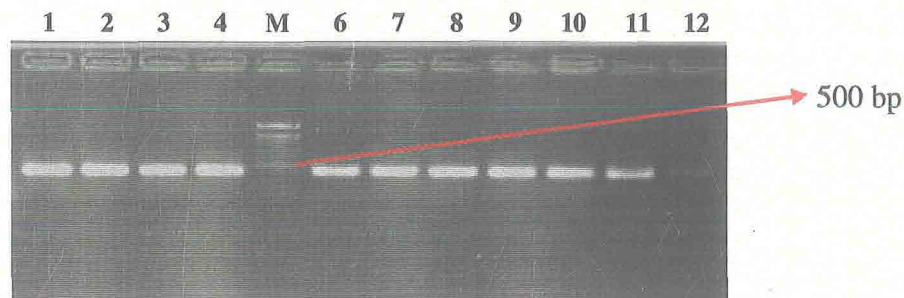


Fig. 4.11 PCR for confirmation of *atp6* RACE product

Lanes 1 to 4. PCR with RACE product; 9 to 15. Colony PCR with T/A-RACE *atp6* clones.

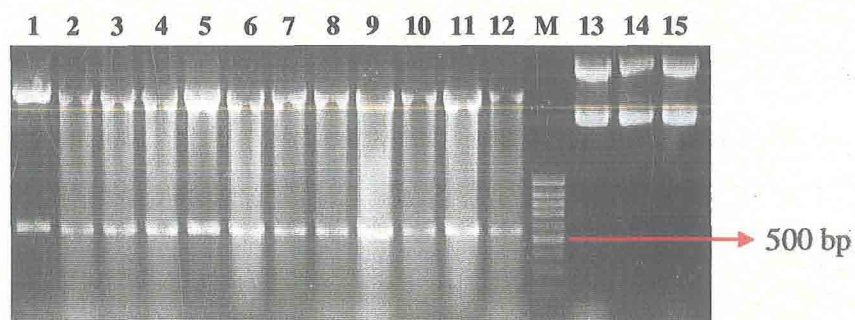


Fig. 4.12 Restriction of T/A-RACE clones with *HindIII* and *EcoRI*

Lanes 1 to 12. T/A-RACE *atp6* clones; 13,14&15. uncut T/A-RACE *atp6* clones; M. 100 bp ladder.



Fig. 4.13 Confirmation of sunflower T/A-*atp6* FIRI cDNA clones

Upper panel: Restriction with *HindIII* (clones with 604 bp band (yellow arrow) are having insert in the sense orientation).

Lower panel: Restriction with *SacI* Lanes (clones with 931 bp band (blue arrow) are having insert in the antisense orientation).

Based on this premise, RNA editing was studied for safflower *nad3* and *atp9* genes, as both are very crucial genes for mitochondrial activity and both are reported to undergo extensive editing in other crops. Primers were designed based on sunflower *atp9* (acc. no. AF258785) and *nad3* (acc. no. X84008) sequence data available online at www.ncbi.nlm.nih.gov.in. Two sets of primers were designed for both the genes (Table 3.8). One set (primers F1 and R1) was for the region immediately flanking the coding region of these genes, so that the RNA editing sites through the entire length of the genes could be detected and the other set (primers F2 and R2) was for the coding region of these genes, so that these genes could be amplified even if the flanking regions for the genes in safflower were not conserved compared to that in sunflower (Figure 4.19 and 4.20).

4.1.7 Cloning of genomic *nad3* and *atp9* sequences of safflower into T/A vector

Initially, to check whether these two gene sequences in safflower were similar to those in sunflower, PCR was set up with the primers designed within the coding regions (F2 and R2) (Figure 4.14). Expected size amplicons were obtained for both *nad3* (357 bp) and *atp9* (261 bp). Then the other set of primers (designed for the flanking region) were tried for these genes. In case of *nad3*, the expected amplicon was obtained and the same was cloned into T/A cloning vector. But in case of *atp9*, no product was obtained when R1 primer was used, indicating that the 3' flanking region of safflower might not be conserved (in comparison to sunflower). Hence, PCR product obtained with F1 and R2 primer was cloned into the T/A vector. The cloning was confirmed by restriction with *Hind*III and *Eco*RI that gave bands of 465 bp for *nad3* and 356 bp for *atp9* (Figure 4.15). The orientation of the insert in T/A vector in the clones were confirmed by digestion with *Sac*I.

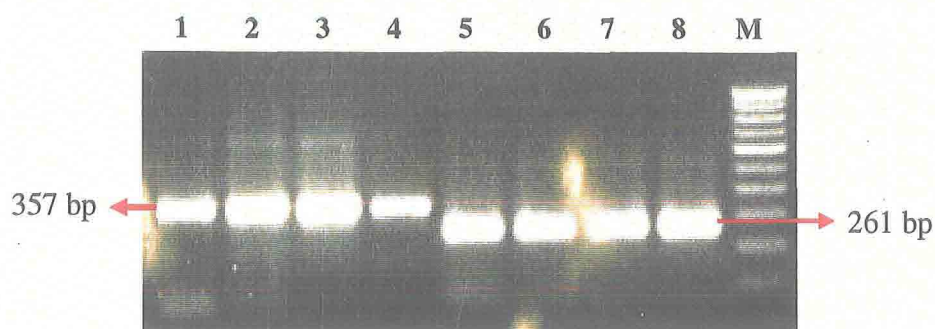


Fig. 4.14 PCR for *nad3* and *atp9* with mitochondrial DNA of safflower and sunflower

Lanes 1 to 4. PCR for *nad3*; 1&2. 1 ng & 5 ng safflower mt DNA; 3&4. 1 ng & 5 ng sunflower mt DNA; 5 to 9. PCR for *atp9*; 5&6. 1 ng & 5 ng safflower mt DNA; 7&9. 1 ng & 5 ng sunflower mt DNA; M. 100 bp DNA ladder.

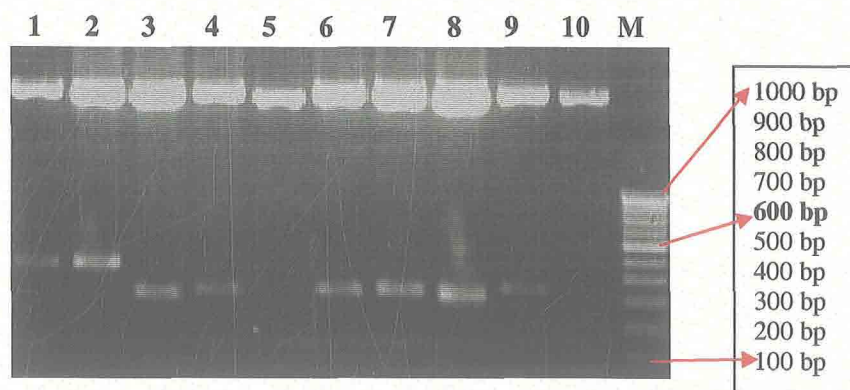


Fig. 4.15 Confirmation of T/A-*nad3* and T/A-*atp9* genomic clones by restriction analysis with *HindIII* and *EcoRI*.

Lanes 1&2. positive T/A genomic *nad3* clones; 3,4,6,7,8&9 positive T/A genomic *atp9* clones; 5&10. negative clones; M. 100 bp ladder. Note the size differences between *nad3* and *atp9* clones. The differences seen within *atp9* clones are due to different plasmid DNA concentrations.

4.1.8 Cloning of cDNA of *nad3* and *atp9* of safflower into T/A vector

RT-PCR was done using safflower mitochondrial RNA for both these genes using R1 and F1 primers. RT-PCR amplicon size matched exactly with the PCR amplicon size for both *nad3* (357bp) and *atp9* (261bp), indicating the absence of introns for these two genes in safflower (Figure 4.16). The RT-PCR amplicons for these genes were cloned into T/A vector. The cloning was confirmed by restriction with *HindIII* and *EcoRI* that gave products of expected size (Figure 4.17 and 4.18). The orientation of the insert in T/A vector in the clones was confirmed by digestion with *SacI*.

4.1.9 *In silico* analysis of *nad3* and *atp9* gene and cDNA as well as the deduced protein sequences

A comparison of the genomic and cDNA clones revealed extensive editing of the transcripts of both the genes, all of which were C to U conversions (Figure 4.19 and 4.20). In order to avoid considering errors that could have occurred during sequencing or PCR, only such changes that occurred in at least three cDNA clones were considered as edit sites for further analysis. In the present investigation, the sites which showed editing in all the sequenced clones were designated as complete edit sites while the sites that did not show editing in all but in at least three clones, were designated as partial edit sites.

4.1.9.1 RNA editing in *nad3* and *atp9* transcripts of safflower

In case of *nad3*, 19 C-U changes were seen in more than three clones and hence were considered for further analysis. Of these, 12 sites were edited consistently in all the clones (complete edit sites) whereas 7 were partial edit sites (Figure 4.21; Table 4.2). A total of 14



Fig. 4.16 RT-PCR for *nad3* and *atp9* of safflower

Lanes 1 to 3. RT-PCR for *nad3* (with dilutions of RT product); 4. Genomic-PCR for *nad3*; 5. Treated RNA-PCR for *nad3* and *atp9*; 6. Untreated RNA-PCR for *nad3* and *atp9*; 7. Blank-PCR for *nad3* and *atp9* (without DNA or RNA); 8-10. RT-PCR for *atp9*; 11. Genomic-PCR for *atp9*.

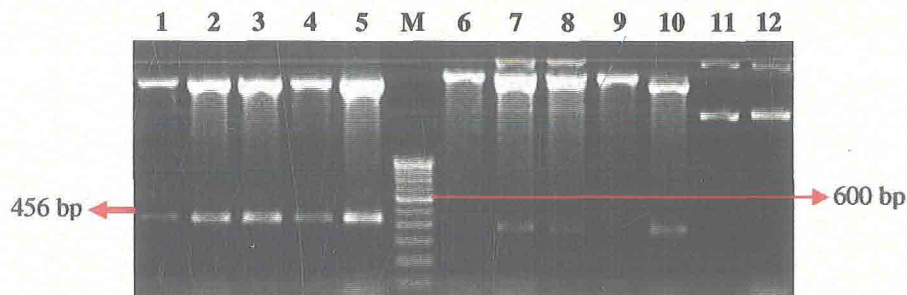


Fig. 4.17 Confirmation of T/A-*nad3* cDNA clones

Lanes 1 to 5. Restriction of clones with *Hind*III and *Eco*RI; 6 to 10. Restriction of same clones with *Sac*I; M. 100 bp DNA ladder; 11&12. uncut clones
Bands in lanes 7, 8 & 10 indicate clones with the 5'-3' orientation of insert whereas lanes 6 & 9 indicate clones with insert in the 3'-5' orientation.



Fig. 4.18 Confirmation of T/A-*atp9* cDNA clones

Lanes 1 to 10. Restriction with *Hind*III and *Eco*RI;
M. 100 bp DNA ladder.

Table 4.2 Summary of RNA editing and encoded amino acid changes for *nad3* gene of safflower

Edit site no.	Partial/complete editing (PE/CE)	Relative Editing Efficiency (REE)	Nucleotide position	Codon position	Amino acid change	Predicted by PREP-Mt Yes/No
1	CE	1.0	5 ^A	2 ^A	TCA(S)-TTA(L)	Yes
2	CE	1.0	39 ^A	13 ^A	ATC(I)-ATT(I)	No
3	CE	1.0	44 ^A	15 ^A	CCG(P)-CTG(L)	Yes
4	CE	1.0	61 ^A	21 ^A	CCA(P)-TTA(L)	No
5	PE	0.9	62 ^A			Yes
6	CE	1.0	80 ^A	27 ^A	CCA(P)-CTA(L)	Yes
7	PE	0.3	147	49 ^A	TTC(F)-TTT(F)	No
8	CE	1.0	208 ^A	70 ^A	CCT(P)-TTT(F)	Yes
9	CE	1.0	209 ^A			Yes
10	CE	1.0	215 ^A	72 ^A	CCG(P)-CTG(L)	Yes
11	PE	0.8	230 ^A			No
12	PE	0.7	231 ^A	77 ^A	TCC(S)-TTT(F)	Yes
13	PE	0.8	247 ^A	83 ^A	CCT(P)-TCT(S)	Yes
14	CE	1.0	251 ^A	84 ^A	CCC(P)-CTC(L)	Yes
15	CE	1.0	266 ^A	89 ^A	CCC(P)-CTC(L)	Yes
16	PE	0.9	275 ^A	92 ^A	TCT(S)-TTT(F)	Yes
17	CE	1.0	317 ^A	106 ^A	TCT(S)-TTT(F)	Yes
18	PE	0.9	344 ^A	115 ^A	TCG(S)-TTG(L)	Yes
19	CE	1.0	349 ^A	117 ^A	CGG(R)-TGG(W)	Yes

REE = number of clones showing editing /total number of clones (12)

CE: Complete edited site (edited in all clones)

PE: Partial edited site (not edited in all clones)

^A - edited nucleotide/edited aminoacid conserved in the majority of the species

[illegible]

Fig. 4.19. Comparison of genomic and cDNA sequences of *nad3* gene of safflower. The second and the third lines indicate the genomic and the cDNA sequences respectively. The corresponding amino acid sequences are given in the first and the fourth line. Edited sites are given in bold and boxes depict them along with the corresponding codon and amino acid changes. The primer sequences are indicated by grey shade. Stop codon is indicated by *.

amino acid substitutions were seen due to editing, the most common being Proline to Leucine (P-L) (6 changes). The other changes were Serine to Phenylalanine (S-F) (three changes), Serine to Leucine (S-L) (two changes) and one each of Proline to Phenylalanine (P-F), Arginine to Tryptophan (R-W) and Proline to Serine (P-S) (Figure 4.19; Table 4.2). Of the 12 clones studied, only 4 (clones 2, 5, 6 and 11) had all the sites edited (Figure 4.21).

A total of 15 differences between the genomic and cDNA sequences encoding *atp9* were observed, of which 12 were seen in more than three clones and hence considered for further studies. Of these 12 edit sites, 10 were complete edit sites with 8 leading to amino acid substitutions. The most common amino acid substitution was S-L (5 changes) while one each of P-L, S-F and L-F were seen. Of the remaining two completely edited sites, one at nucleotide position 81 did not lead to any amino acid change while the other at nucleotide position 223 modified an Arginine (A) codon (CGA) to a termination codon (TGA). Interestingly, the two partial edit sites at codon 30 and 69 observed in safflower were silent edit sites (not leading to amino acid changes) as reported in other species. Among the 11 clones studied, only four clones (1, 5, 7 and 9) were edited at all the 12 edit sites (Figure 4.22; Table 4.3). The genomic and cDNA (completely edited) sequences obtained in this study have been submitted to GenBank (*nad3* gene-DQ539623; *atp9* gene-DQ539624; *nad3* mRNA DQ534204; *atp9* mRNA DQ534203).

A comparison of the editing in these genes, with those reported in other angiosperms revealed that the sites were conserved sites and were reported in many species (Figure 4.23 and 4.24; Tables 4.2 and 4.3).

The *nad3* and *atp9* genomic sequences of safflower obtained in this study were analyzed using PREP-Mt (<http://www.prep-mt.net/>), a software for prediction of edited sites

Table 4.3 Summary of RNA editing and encoded amino acid changes for *atp9* gene of safflower

Edit site no.	Partial/complete editing (PE/CE)	Relative Editing Efficiency (REE)	Nucleotide position	Codon position	Amino acid change	Predicted by PREP-Mt Yes/No
1	CE	1.0	50 ^A	17 ^A	TCA(S)-TTA(L)	Yes
2	CE	1.0	81 ^A	27 ^A	GTC(V)-GTT(V)	No
3	CE	1.0	82 ^A	28 ^A	CTT(L)-TTT(F)	Yes
4	PE	0.5	90 ^A	30 ^A	TCC(S)-TCT(S)	No
5	CE	1.0	92 ^A	31 ^A	TCG(S)-TTG(L)	Yes
6	CE	1.0	134 ^A	45 ^A	TCA(S)-TTA(L)	Yes
7	CE	1.0	182 ^A	61 ^A	TCG(S)-TTG(L)	Yes
8	CE	1.0	191 ^A	64 ^A	CCA(P)-CTA(L)	Yes
9	PE	0.8	205 ^A	69 ^A	CTG(L)-TTG(L)	No
10	CE	1.0	212 ^A	71 ^A	TCA(S)-TTA(L)	Yes
11	CE	1.0	215 ^A	72 ^A	TCC(S)-TTC(F)	Yes
12	CE	1.0	223 ^A	75 ^A	CGA(R)-TGA(^A)	Yes

REE = number of clones showing editing /total number of clones (11)

CE: Complete edited site (edited in all clones)

PE: Partial edited site (not edited in all clones)

^A - edited nucleotide/edited aminoacid conserved in the majority of the species

	5♦	15	25	35♦	45♦	55	65♦	75♦	85	95	105♦	115
genomic	ATGTCAGAAT	TTGCACCTAT	TTTATCTAT	TTAGTGATCA	GTCCGCTAGT	TTCTTTTGATC	CCACTCGGTG	TTCCCTTTCC	ATTGCTTCC	AAATGTTCA	CCTATCCAGA	AAATGTTCCG
cdNA1	T			T	T	TT	TT	T			T	
cdNA 2	T			T	T	TT	TT	T				
cdNA 3	T			T	T	TC	TC	T				
cdNA 4	T			T	T	TT	TT	T				
cdNA 5	T			T	T	TT	TT	T				
cdNA 6	T			T	T	TT	TT	T				
cdNA 7	T			T	T	TT	TT	T				
cdNA 8	T			T	T	TT	TT	T				
cdNA 9	T			T	T	TT	TT	T				
cdNA 10	T			T	T	TT	TT	T				
cdNA 11	T			T	T	TT	TT	T				
cdNA 12	T			T	T	TT	TT	T				
genomic	GCCTACGAAT	GTGGTTTCGA	TCCTTTCCGT	GATGCCAGAA	GTGCTTTTGA	TATACGATTT	TATCTTGT	CTATTTTAT	TATTATCCT	GATCCGGAG	TCACCTTTTC	CTTCTCTGG
cdNA 1	T			C					TT	T		T
cdNA 2	T			T					TT	T		T
cdNA 3	T			C					TT	T		C
cdNA 4	T			C				C	TT	T		T
cdNA 5	T			T					TT	T		T
cdNA 6	T			T					TT	T		T
cdNA 7	T			C					TT	T		T
cdNA 8	T			C					TT	T		T
cdNA 9	T			C					TT	T		C
cdNA 10	T			C					TT	T		T
cdNA 11	T			T					TT	T		T
cdNA 12	T			C					TT	T		C
genomic	GCAGTACCTC	CCAACAAGAT	TGATCCCTTT	GGATCTTGGT	CCATGATGGC	CTTTTATATG	ATTTTGACGA	TTGGATCTCT	CTATGAATGG	AAAAGGGGTG	CTTCGGATCG	GGAGTAA
cdNA 1	T	T		T				T			T	TAA
cdNA 2	T	T		T				T			T	TAA
cdNA 3	C	T		T				T			T	TAA
cdNA 4	T	T		T				T			T	TAA
cdNA 5	T	T		T				T			T	TAA
cdNA 6	T	T		T				T			T	TAA
cdNA 7	T	T		T				T			T	TAA
cdNA 8	T	T		T				T			T	TAA
cdNA 9	C	T		C				T			C	TAA
cdNA 10	T	T		T				T			T	TAA
cdNA 11	T	T		T				T			T	TAA
cdNA 12	T	T		T				T			T	TAA

Fig. 4.21. Editing in different cDNA clones of *nad3*. The numbers on the top line indicate the nucleotide positions. The sequence given at the top is the genomic sequence and below that the cDNA sequences are provided. ♦ indicates completely edited sites (found to be edited in all the cDNA clones). V indicates partially edited sites (edited in more than three but not all the cDNA clones). ◊ indicates changes found in less than three cDNA clones and hence not considered as edited sites for the present study.

	5	15	25	35	45	55	65	75	85	95
genomic	ATGTTAGAAG	GTGCAAAACT	AATAGGGGCC	GGAGCTGCTA	CAATTGCTTC	AGCGGAGCT	GCTATCGGTA	TTGGAACGT	CCTTAGTTCC	TGGATTCAAT
cdNA 1					T				TT	
cdNA 2					T				TT	
cdNA 3					T				TT	
cdNA 4					T				TT	
cdNA 5					T				TT	
cdNA 6					T				TT	
cdNA 7					T				TT	
cdNA 8					T				TT	
cdNA 9					T				TT	
cdNA 10					T				TT	
cdNA 11					T				TT	
genomic	105	115	125	135	145	155	165	175	185	195
	CCGTGGCTCG	AAATCCATCA	TTGGCTAAAC	AATCATTTGG	TTATGCCATT	TTAGGCTTTC	CTCTAACCGA	AGCTATCGCA	TCGTTTGCCC	CAATGATGGC
cdNA 1										
cdNA 2										
cdNA 3										
cdNA 4										
cdNA 5										
cdNA 6										
cdNA 7										
cdNA 8										
cdNA 9										
cdNA 10										
cdNA 11										
genomic	V205	215	225	235	245	255				
	CTTTCGTGATC	TCATCCGAT	TCCGATCAA	GAATCAAAGA	AAGAAGGTTG	AAGTTTCATA	A			
cdNA 1										
cdNA 2										
cdNA 3										
cdNA 4										
cdNA 5										
cdNA 6										
cdNA 7										
cdNA 8										
cdNA 9										
cdNA 10										
cdNA 11										

Fig. 4.22. Editing in different cDNA clones of *atp9*: The numbers on the top line indicate the nucleotide positions. The sequence given at the top is the genomic *atp9* sequence and below this the cDNA sequences are provided. ♦ indicates completely edited sites (found to be edited in all the cDNA clones) ▽ indicates partially edited sites (edited in more than three but not in all the cDNA clones) ◊ indicates changes found in less than three cDNA clones and hence not considered as edited sites for the present study.

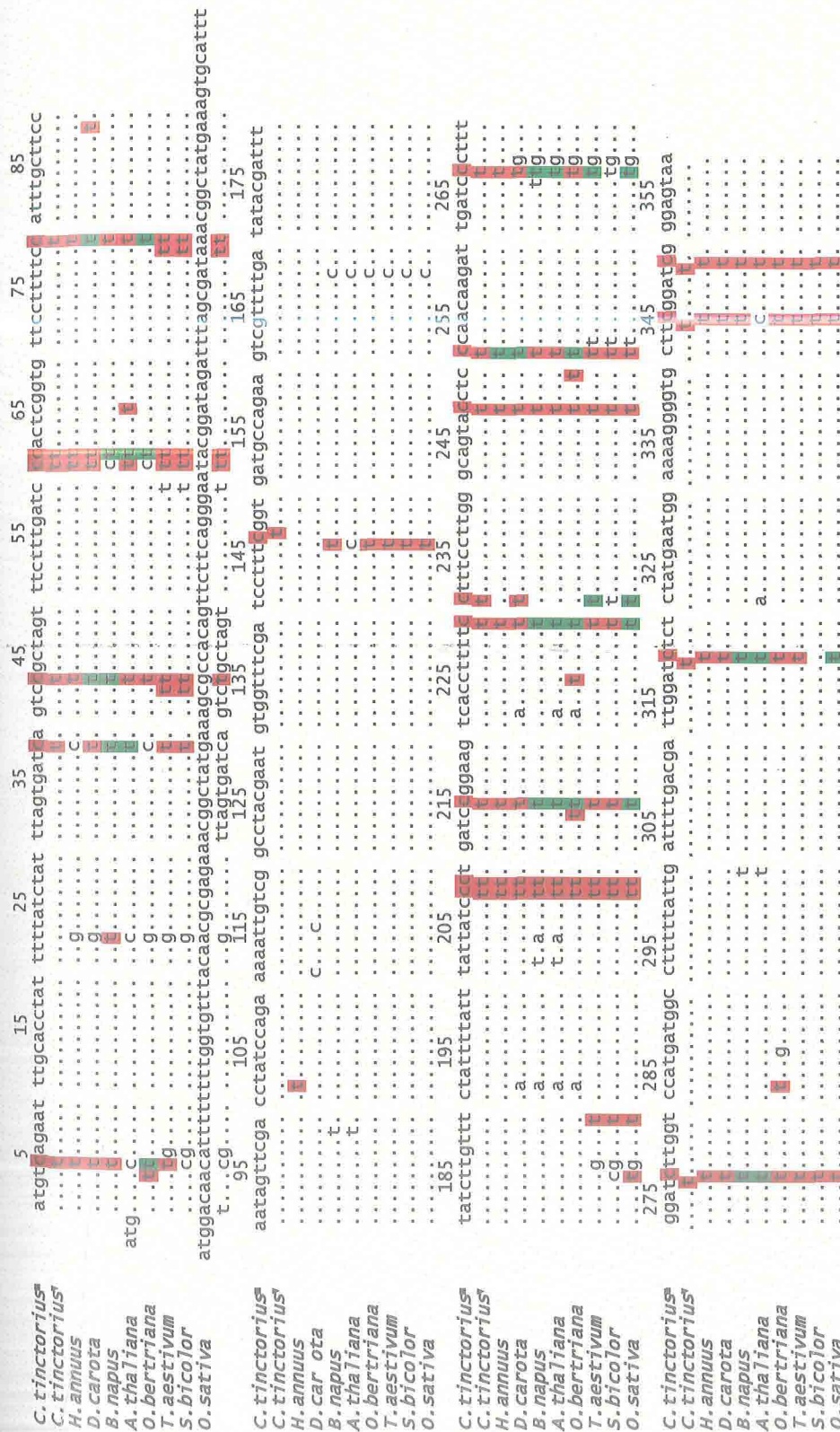


Fig. 4.23. Comparison of *nad3* genomic and cDNA sequences of *C. tinctorius* with cDNA sequences of other species (g) indicates genomic sequence whereas all others are the cDNA sequences. Dots indicate similarity to *C. tinctorius* genomic sequence (first line). Except for *D. carota* and *O. bertriana*, all other cDNA sequences were deduced from gene sequences based on the reported edit sites. t indicates an edited 't' in the mRNA. g indicates genomically encoded 't'. t indicates a potential site which gets edited in mRNA. *H. annuus* (X84008), *D. carota* (mRNA AJ300556), *B. napus* (AP006444), *A. thaliana* (NC001284), *O. bertriana* (mRNA X52200), *T. aestivum* (X59153), *S. bicolor* (Z85978), *O. sativa* (BA000029). The *Carthamus tinctorius* genomic^a and cDNA^b (from a completely edited clone) sequences reported in this study have been submitted to GenBank.

(Mower 2005). Using this software, it was possible to predict 16 (11 complete and 5 partial) out of the total 19 edit sites of *nad3* and all the 9 complete edit sites (out of the total 12 edit sites) of *atp9* (Tables 4.2 and 4.3).

4.1.9.2 Analysis of the deduced protein sequences

As editing is only an intermediary step in the process of formation of functional proteins, the actual effect of editing will have to be assessed at the protein level. A comparison of amino acid sequence derived from genomic as well as cDNA sequences of safflower along with cDNA derived amino acid profiles of other species reported earlier, clearly indicated that editing in these genes of safflower led to formation of conserved amino acids (Figures 4.25 and 4.26; Tables 4.2 and 4.3). This was clearly evident in case of ATP9 where the length of protein was reduced due to editing in a conserved manner across species. Hydrophobicity analysis of the polypeptides derived from both genomic and cDNA sequences of safflower demonstrated that the editing resulted in increased hydrophobicity as indicated by the elevated scores (in the Y axis) at the peaks in Figures 4.27 and 4.28. Analysis with the transmembrane prediction software (HMMTOP) indicated that in *nad3* encoded protein, editing led to increased number of amino acids being included in the second and third transmembrane helices with concomitant reduction in the outer loop between the two (Figure 4.29). However, though editing did not lead to change in the structure of the transmembrane helices of the ATP9 protein, there was a reduction (by twelve amino acids) in the second inner tail (at the carboxy terminal) owing to the creation of a stop codon by editing (Fig. 4.30).

<i>C. tinctorius</i> ^a	5	15	25	35	45	55	65	75	85	95	105	115						
<i>C. tinctorius</i> [▼]	MSEFAPFIY	LV	SLVSLI	LGVPF	FAS	NSSTYPEKLS	AYECGFDP	G	DARSRFDIR	YLVSLFII	D	EVTF	FPW	AVSNKIDF	G	WSMMAFLIIITIG	LYEWKRGAD	DE*
<i>H. annuus</i>	MSEFAPFIY	LV	SLVSLI	LGVPF	FAS	NSSTYPEKLS	AYECGFDP	G	DARSRFDIR	YLVSLFII	D	EVTF	FPW	AVSNKIDF	G	WSMMAFLIIITIG	LYEWKRGAD	DE*
<i>D. carota</i>	MSEFAPICIY	LV	SLVSLI	LGVPF	FAS	NSSTYPDKLS	AYECGFDP	G	DARSRFDIR	YLVSLFII	D	EVTF	FPW	AVSNKIDF	G	WSMMAFLIIITIG	LYEWKRGAD	DE*
<i>B. napus</i>	MSEFAPFIY	LV	SLVSLI	LGVPF	FAS	NSSTYPDKLS	AYECGFDP	G	DARSRFDIR	YLVSLFII	D	EVTF	FPW	AVSNKIDF	G	WSMMAFLIIITIG	LYEWKRGAD	DE*
<i>A. thaliana</i>	MSEFAPISIIY	LV	SLVSLI	LGVPF	FAS	NSSTYPEKLS	AYECGFDP	G	DARSRFDIR	YLVSLFII	D	EVTF	FPW	AVSNKIDF	G	WSMMAFLIIITIG	LYEWKRGAD	DE*
<i>O. bertriana</i>	MSEFAPICIS	LV	SLVSLI	LVVPF	FSS	NSSTYPEKLS	AYECGFDP	SG	DARSRFDIR	YLVSLFII	D	EVTF	FPW	AVSNKIDF	G	WSMMAFLIIITIG	LYEWKRGAD	DE*
<i>T. aestivum</i>	MSEFAPICIY	LV	SLVSLI	LGVPF	FAS	NSSTYPEKLS	AYECGFDP	SG	DARSRFDIR	YLVSLFII	D	EVTF	FPW	AVSNKIDF	G	WSMMAFLIIITIG	LYEWKRGAD	DE*
<i>S. bicolor</i>	MSEFAPICIY	LV	SLVSLI	LGVPF	FAS	NSSTYPEKLS	AYECGFDP	SG	DARSRFDIR	YLVSLFII	D	EVTF	FPW	AVSNKIDF	G	WSMMAFLIIITIG	LYEWKRGAD	DE*
<i>O. sativa</i>	MDNIFFGVNARNGYESATV	LOGIRID	LAINGESAF															
	LSEFAPICIY	LV	SLVSLI	LGVPF	FAS	NSSTYPEKLS	AYECGFDP	SG	DARSRFDIR	YLVSLFII	D	EVTF	FPW	AVSNKIDF	G	WSMMAFLIIITIG	LYEWKRGAD	DE*

Fig. 4.25 Comparison of the NAD3 protein deduced from the genomic and mRNA sequences of *C. tinctorius* (present study) with that of mRNA sequences of other species. *H. annuus* (X84008), *D. carota* (mRNA AJ300556) *B. napus* (AP006444), *A. thaliana* (NC001284), *O. bertriana* (X52200), *T. aestivum* (X59153), *S. bicolor* (Z85978), *O. sativa* (BA000029). In all the species (except *O. bertriana* and *D. carota*), the edited mRNA sequences were obtained by translation from the genomic sequences based on the reported edit sites. Note the additional sequences found on the N-terminal side of rice and *Arabidopsis*. The *C. tinctorius* protein sequences deduced from *nad3* gene^a and cDNA[▼].

	5	15	25	35	45	55	65	75				
<i>C. tinctorius</i> ^a	MLEGAKLIGA	GAATIA	AGA	AIGIGNVLS	SIHSVARNPS	LAKQ	FGYAI	LGFALTEAIA	SPAPMAFLI	SSVF	SKNQR	KKVEVS*
<i>C. tinctorius</i> [▼]	MLEGAKLIGA	GAATIA	AGA	AIGIGNVFS	SIHSVARNPS	LAKQ	FGYAI	LGFALTEAIA	LPALMMAFLI	LPVF*		
<i>B. napus</i>	MLEGAKLIGA	GAATIA	AGA	AIGIGNVFS	SIHSVARNPS	LAKQ	FGYAI	LGFALTEAIA	LPALMMAFLI	LPVF*		
<i>N. tabacum</i>	MLEGAKLIGA	GAATIA	AGA	AIGIGNVFS	SIHSVARNPS	LAKQ	FGYAI	LGFALTEAIA	LPALMMAFLI	LPVF*		
<i>A. thaliana</i>	MTKREYNSQ	MLEGAKLIGA	GAATIA	AGA	AIGIGNVFS	SIHSVARNPS	LAKQ	FGYAI	LGFALTEAIA	LPALMMAFLI	LPVF*	
<i>G. max</i>	MLEGAKLIGA	GAATIA	AGA	AVGIGNVFS	SIHSVARNPS	LAKQ	FGYAI	LGFALTEAIA	LPALMMAFLI	LPVF*		
<i>O. sativa</i>	MLEGAKLIGA	GAATIA	AGA	AVGIGNVFS	SIHSVARNPS	LAKQ	FGYAI	LGFALTEAIA	LPALMMAFLI	LPVF*		
<i>T. aestivum</i>	MLEGAKLIGA	GAATIA	AGA	AVGIGNVFS	SIHSVARNPS	LAKQ	FGYAI	LGFALTEAIA	LPALMMAFLI	LPVF*		
<i>Z. mays</i>	MLEGAKLIGA	GAATIA	AGA	AVGIGNVFS	SIHSVARNPS	LAKQ	FGYAI	LGFALTEAIA	LPALMMAFLI	LPVF*		

Fig. 4.26 Comparison of ATP9 protein deduced from the genomic and mRNA sequences of *C. tinctorius* (present study) with that of mRNA sequences of other species. Sequences were translated to obtain the corresponding protein sequences. *B. napus* (D13696), *N. tabacum* (mRNA X64423), *A. thaliana* (001284), *G. max* (L17319), *O. sativa* (BA000029), wheat (mRNA X54621), *Z. mays* (mRNA AF390542), soybean (mRNA L17319). In *B. napus*, *A. thaliana* and *O. sativa* the edited mRNA sequences were obtained by translation from the genomic sequences based on the reported edit sites. Note the additional sequences found on the N-terminal side of *Arabidopsis*. The *Carthamus tinctorius* protein sequences deduced from *nad3* gene^a and cDNA[▼].

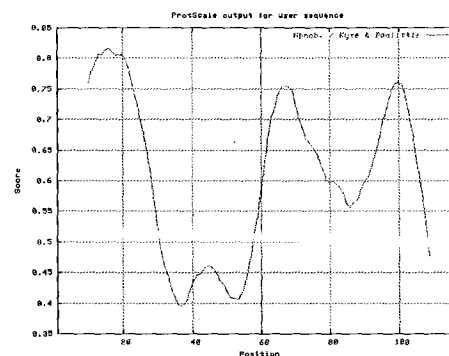
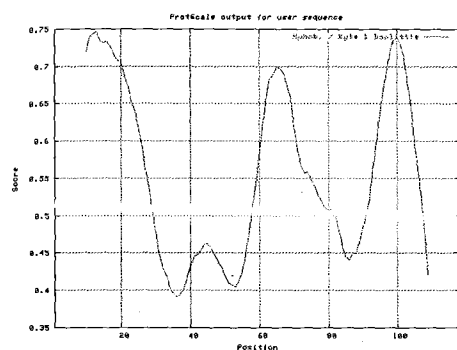


Fig 4.27 Genomically encoded - unedited (left) and cDNA encoded - edited (right) NAD3 protein analysed by ProtScale using Kyte and Doolittle Scales for Hydrophobicity. Note the increased hydropathy scores in case of the edited protein.

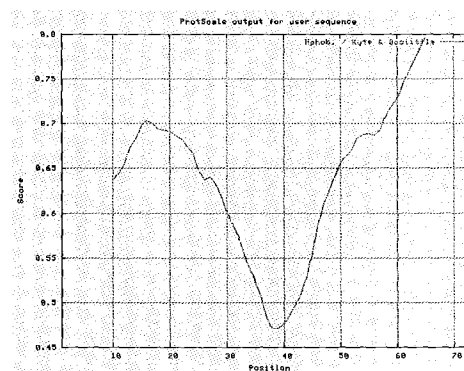
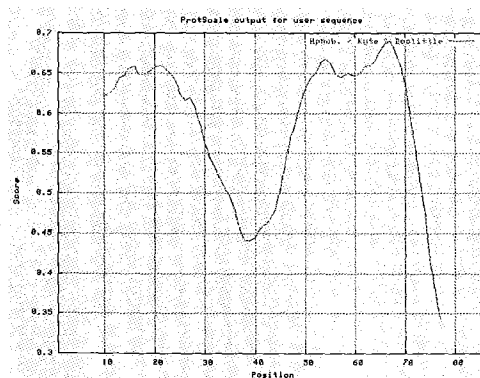


Fig 4.28 Genomically encoded - unedited (left) and cDNA encoded - edited (right) ATP9 protein analysed by ProtScale using Kyte and Doolittle Scales for Hydrophobicity. Note the increased hydropathy scores in case of the edited protein especially at the carboxy terminal.

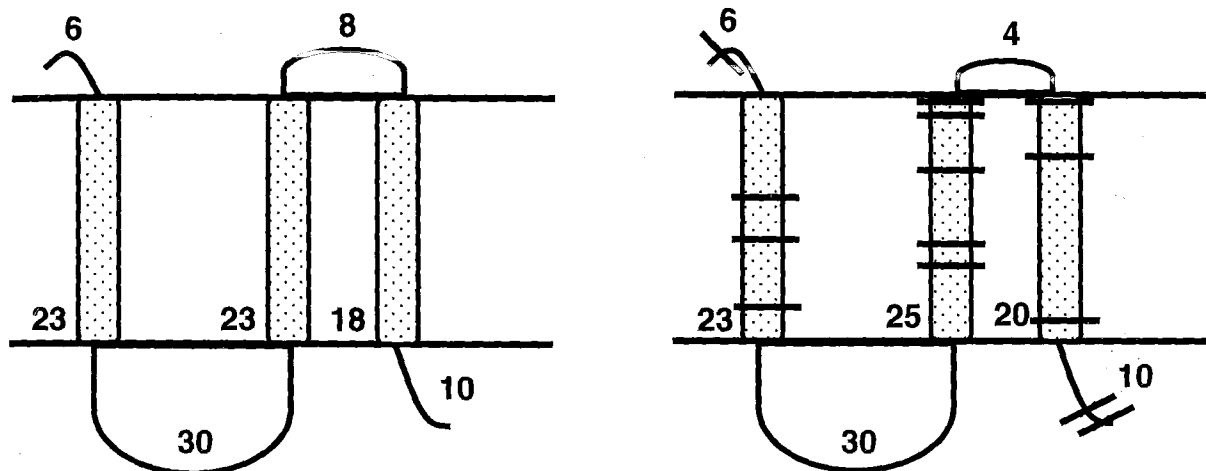


Figure 4.29 Diagrammatic representation of the transmembrane helices predicted by HMMTOP for a) unedited NAD3 b) edited NAD3. Numbers indicate the total aminoacid residues in the helices/tails.

- site of aminoacid change due to editing
- ▤ transmembrane helix
- ⤵ tail portion

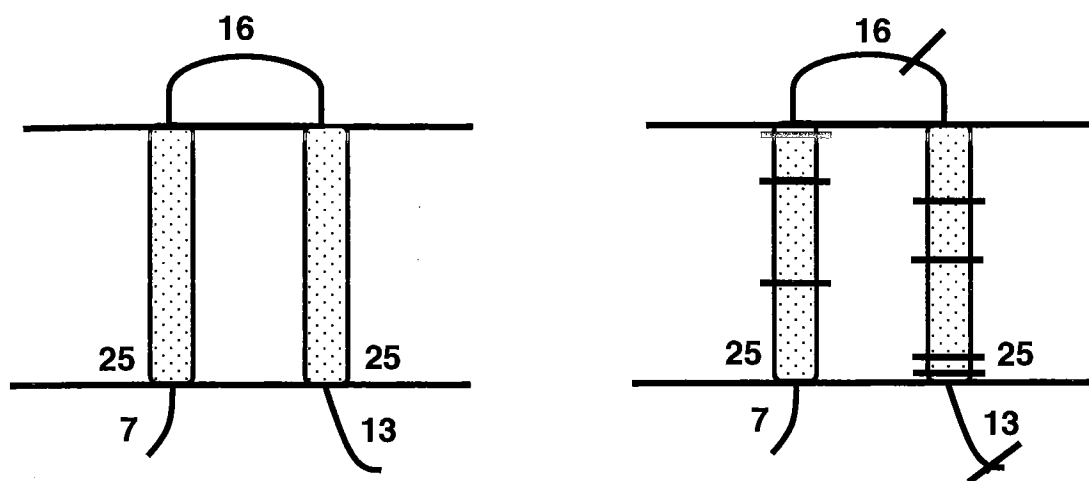


Figure 4.30 Diagrammatic representation of the transmembrane helices predicted by HMMTOP for a) unedited ATP9 b) edited ATP9. Numbers indicate the total aminoacid residues in the helices/tails.

- site of aminoacid change due to editing
- ▤ transmembrane helix
- ⌋ tail portion

These results indicated that substantial RNA editing in *nad3* and *atp9* transcripts along with several aminoacid changes in the deduced proteins leads to increased hydrophobicity making these genes appropriate candidates for induction of transgenic male sterility. Hence these two genes were used for development of constructs for male sterility induction as well as for the development of corresponding constructs for restoration of fertility.

4.2 Standardizing the shoot regeneration in safflower through embryogenic pathway

Regeneration of shoots from transformed tissues is essential in order to develop transgenic plants. Hence, experiments were conducted to develop protocols for efficient regeneration from safflower explants. In the present investigation, induction of shoots using immature embryo explants of safflower, was attempted as immature embryos have been suggested as suitable material for *de novo* shoot induction.

4.2.1 Response in the primary media containing TDZ/ TDZ+NAA

Six media combinations using different concentrations of TDZ with or without NAA (1mg/l) were tried (E₁: MS + 3% sucrose + TDZ 0.5 mg/l + NAA 1.0 mg/l, E₂: MS + 10% sucrose + TDZ 0.5 mg/l + NAA 1.0 mg/l, E₃: MS + 3% sucrose + TDZ 0.5 mg/l, E₄: MS + 10% sucrose + TDZ 0.5 mg/l, E₅: MS + 3% sucrose + TDZ 1.0 mg/l, E₆: MS + 10% sucrose + TDZ 1.0 mg/l) (Table 3.10). Immature embryos isolated from surface sterilized immature seeds were inoculated on these primary media combinations and observations were recorded 15 days after incubation (Tables 4.4). In almost all media combinations, majority of the explants developed into whole seedlings (Plate 4.1). However, explants on three media

Table 4.4 Response of immature embryos on primary media containing different concentrations of TDZ with or without NAA

Code	Media combination	Total no. of explants	Percentage of explants giving rise to					
			Whole seedlings	Embryo- genic calli	Leafy structures	Somatic embryos	Embryo like structures	Only calli
E ₁	MS + 3% sucrose + TDZ 0.5 mg/l + NAA 1.0 mg/l	148	35 (23.7)	91 (61.5)	3 (2.0)	0 (0)	20 (13.5)	6 (4.1)
E ₂	MS + 10% sucrose + TDZ 0.5 mg/l + NAA 1.0 mg/l	87	16 (18.4)	39 (44.8)	0 (0)	15 (17.2)	0 (0)	5 (5.8)
E ₃	MS + 3% sucrose + TDZ 0.5 mg/l	58	22 (37.9)	15 (25.9)	2 (3.5)	19 (32.8)	0 (0)	0 (0)
E ₄	MS + 10% sucrose + TDZ 0.5 mg/l	107	22 (20.6)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
E ₅	MS + 3% sucrose + TDZ 1.0 mg/l	51	2 (3.9)	17 (33.3)	7 (13.7)	19 (37.3)	2 (3.9)	4 (7.8)
E ₆	MS + 10% sucrose + TDZ 1.0 mg/l	87	48 (55.2)	0 (0)	0 (0)	6 (6.9)	0 (0)	0 (0)



Plate 4.1 Response of the immature embryo explants on E5 medium- developing into whole seedlings

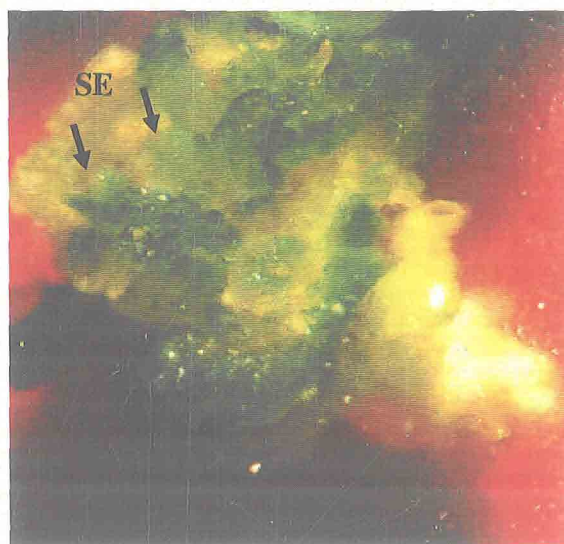


Plate 4.2 Immature embryos giving rise to somatic embryos (SE) along with multiple shoots on E3 medium

combinations E₂, E₃ and E₅ produced substantial embryogenic calli along with somatic embryos (Plate 4.2) and E₁ showed higher amount of embryogenic calli along with embryo like structures (Plate 4.3). The remaining two combinations (E₄ and E₆) gave no or very negligible response.

4.2.2 Response in the primary media containing 2,4-D

Twelve media combinations using different concentrations of 2,4-D were tried (Table 3.10). Five combinations (E₇: MS + 3% sucrose + 2,4-D 0.05 mg/l, E₈: MS + 3% sucrose + 2,4-D 0.2 mg/l, E₁₆: MS + 12% sucrose + 2,4-D 0.05 mg/l, E₁₇: MS+12% sucrose + 2,4-D 0.2 mg/l, E₁₈: MS + 12% sucrose + 2,4-D 0.5 mg/l) did not show any response at all. Explants on E₉ (MS + 3% sucrose + 2,4-D 0.5 mg/l) gave more somatic embryos and few embryogenic calli or embryo-like structures. Three combinations (E₁₀: MS + 6% sucrose + 2, 4-D 0.05 mg/l, E₁₁: MS + 6% sucrose + 2, 4-D 0.2 mg/l, E₁₂: MS + 6% sucrose + 2, 4-D 0.5mg/l) gave calli which were non-responsive. E₁₃ (MS + 9% sucrose + 2, 4-D 0.05 mg/l), E₁₄ (MS + 9% sucrose + 2, 4-D 0.2 mg/l) and E₁₅ (MS + 9% sucrose + 2, 4-D 0.5 mg/l) gave whole seedlings in majority of the explants (Table 4.5). With these preliminary results, it could be concluded that MS basal media having TDZ with or without NAA was better than MS media with 2, 4-D combinations for induction of embryogenic calli from immature embryo explants of safflower.

4.2.3 Response in the Secondary Media

The explants from the primary media were transferred to the secondary media with the objective of elongation of shoot initials/development of embryos to obtain complete

Table 4.5 Response of immature embryos on primary media containing different concentrations of 2,4-D

Code	Media combination	Total no. of explants	Percentage of explants giving rise to					Only calli
			Whole seedlings	Embryo- enic calli	Leafy structures	Somatic embryos	Embryo like structures	
E ₇	MS + 3% sucrose + 2,4-D 0.05 mg/l	45	0	0	0	0	0	0
E ₈	MS + 3% sucrose + 2,4-D 0.2 mg/l	56	0	0	0	0	0	0
E ₉	MS + 3% sucrose + 2,4-D 0.5 mg/l	55	0	2 (3.7)	6 (10.9)	17 (30.9)	0	4 (7.3)
E ₁₀	MS + 6% sucrose + 2,4-D 0.05 mg/l	25	2 (8)	4 (16)	0	0	0	14 (56)
E ₁₁	MS + 6% sucrose + 2,4-D 0.2 mg/l	28	0	0	0	0	0	27 (96.4)
E ₁₂	MS + 6% sucrose + 2,4-D 0.5 mg/l	29	0	0	0	0	0	20 (69.0)
E ₁₃	MS + 9% sucrose + 2,4-D 0.05 mg/l	53	29 (54.7)	0	17 (32.1)	4 (7.6)	3 (5.7)	0
E ₁₄	MS + 9% sucrose + 2,4-D 0.2 mg/l	45	13 (28.9)	5 (11.1)	10 (22.2)	2 (4.4)	8 (17.8)	0
E ₁₅	MS + 9% sucrose + 2,4-D 0.5 mg/l	75	70 (93.3)	0	1 (1.3)	0	1 (1.3)	0
E ₁₆	MS + 12% sucrose + 2,4-D 0.05 mg/l	65	0	0	0	0	0	0
E ₁₇	MS + 12% sucrose + 2,4-D 0.2 mg/l	41	0	0	0	0	0	0
E ₁₈	MS + 12% sucrose + 2,4-D 0.5 mg/l	38	0	0	0	0	0	0

Percent values are indicated within brackets

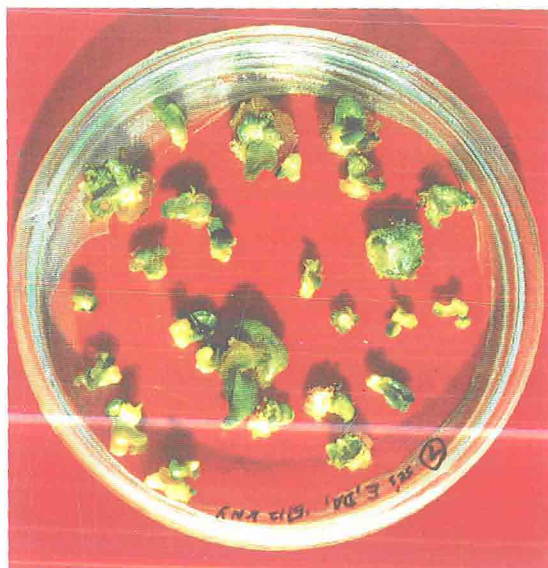


Plate 4.3 Explants showing embryogenic calli and embryo-like structures along with multiple shoots in E1 medium

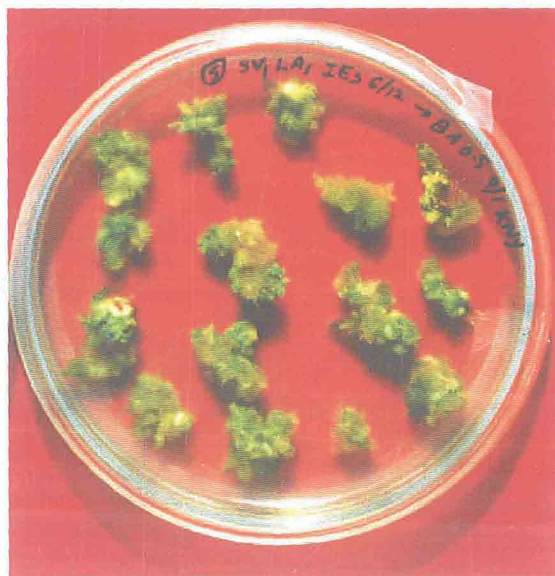


Plate 4.4 Immature embryos cultured on SV1 medium showing formation of multiple shoots

shoots. Since 2, 4-D combinations were non responsive, the four combinations of TDZ (E₁, E₂, E₃ and E₅) with or without NAA, which gave more embryogenic calli along with somatic embryos or embryo like structures, were selected and the explants were transferred to secondary media. MS basal media having 3% sucrose with five different hormonal combinations (S₁-BA 0.2 mg/l, S₂-Kinetin 0.2 mg/l, S₃-Kinetin 0.5 mg/l, S₄-TDZ 0.5 mg/l + NAA 0.1 mg/, S₅- TDZ 0.5 mg/l + NAA 0.5) (Table 3.11) were tried. The response after incubation on these media for 15 days, were recorded. Among the combinations, explants from E₁, E₃ and E₅ when transferred to S₁ or S₂ gave a higher frequency of greenish calli initially from which adventitious shoots arose later. Even among these, the E₁ to S₁ and E₅ to S₁ combinations gave the maximum response. But S₃, S₄ and S₅ were not much responsive though they did show shoot like structures or adventitious shoots in some of the explants (Table 4.6). These observations indicated that the embryogenic calli/embryo like structures/somatic embryos arising on MS medium with 3% sucrose having TDZ 0.5 along with NAA 1.0 or TDZ 1.0 alone when cultured on MS medium with 3% sucrose having BAP 0.2 could give rise to adventitious shoots in higher frequency. Though the aim of this study was induction of somatic embryogenesis, the developed somatic embryos did not continue with normal ontogeny and they did not germinate and instead, the greenish calli yielded adventitious shoots.

4.2.4 Response of different sized immature embryos

As it is reported that stage of the embryos (used as explants) influences the tissue culture response, immature embryos of different sizes were tested for their response to *in vitro* de-differentiation and regeneration. The immature embryos were classified based on

**Table 4.6 Response of immature embryos on five secondary media combinations:
Explants forming adventitious shoots**

Medium	BA 0.2 (S1)	Kinetin 0.2 (S2)	Kinetin 0.5 (S3)	MS + 3% sucrose + TDZ 0.5 mg/l + NAA 0.1 mg/l (S4)	MS + 3% sucrose + TDZ 0.5 mg/l + NAA 0.5 mg/l (S5)
MS + 3% sucrose + TDZ 0.5 mg/l + NAA 1.0 mg/l (E ₁)	13/33 (39.4)	8/38 (21.1)	2/17 (11.8)	0/19 (0)	3/25 (12.0)
MS + 10% sucrose + TDZ 0.5 mg/l + NAA 1.0 mg/l (E ₂)	0/18 (0)	0/30 (0)	2/5 (40.0)	3/31 (9.7)	0/34 (0)
MS + 3% sucrose + TDZ 0.5 mg/l (E ₃)	3/15 (20.0)	9/17 (52.9)	-	5/29 (17.2)	8/37 (21.6)
MS + 3% sucrose + TDZ 1.0 mg/l (E ₅)	18/28 (64.3)	4/7 (57.1)	0/12 (0)	4/28 (14.3)	0/20 (0)

Percent values are indicated within brackets

their sizes into small (<3mm), medium (3 to 5mm) and large (>5mm). Results obtained with above experiments (E₁ and E₅ identified as better primary media and S₁ (BA 0.2 mg/l) as the best secondary media) were used for culturing the different sized embryos.

At the Biotechnology laboratory of DOR, simultaneously efforts were on to develop a regeneration/transformation protocol using safflower axenic seedling explants and two media combinations SV₁ (MS + 3% sucrose + TDZ 1.0 + NAA 0.2 mg/l) and SV₂ (MS + 3% sucrose + TDZ 0.5 + NAA 0.2 mg/l) were identified to be superior for shoot induction. Since the E₁ and E₅ media identified in present study also ultimately led to induction of multiple shoots, it was conjectured to test SV₁ and SV₂ (which are good for seedling explants) also for immature embryo culture. Hence these two media were also tried for culture of large, medium and small safflower immature embryos.

All the combinations ultimately (in the secondary BA 0.2 mg/l medium) gave rise to multiple shoots irrespective of their initial response (somatic embryos/embryo-like structures in case of E₁ and E₅ or greenish calli in case of SV₁ and SV₂). On the whole, medium sized (3 to 5mm) embryos were the best giving more frequency of multiple shoots. SV₁ (Plate 4.4), SV₂ (Plate 4.5) as well as E₁ gave high frequency of multiple shoots, with explants cultured on SV₂ under light giving 100% response (Table 4.7). E₅ gave a higher frequency of whole seedlings (Table 4.7).

Though experiments on *Agrobacterium* mediated transformation using immature embryos as explants, were also carried out (Plates 4.6 and 4.7), they were not used further. However when these experiments were being carried out, in an on-going project at DOR, a transformation and regeneration protocol was developed in the laboratory using seedling explants (Rao *et al.*, 2007). Since use of seedling explants had several advantages like

**Table 4.7 Response of different sized immature embryos on four media combinations:
Explants forming adventitious shoots**

Code	Media combination	Dark			Light		
		Small sized	Medium sized	Large sized	Small sized	Medium sized	Large sized
SV1	MS + 3% sucrose + TDZ 1.0 + NAA 0.2 mg/l	5/11 (45.5)	5/10 (50.0)	0 (0)	6/12 (50.0)	5/7 (71.4)	2/5 (40.0)
SV2	MS + 3% sucrose + TDZ 0.5 + NAA 0.2 mg/l	2/12 (16.7)	4/14 (28.6)	3/14 (21.4)	6/8 (75.0)	4/4 (100)	1/3 (33.3)
E1	MS + 3% sucrose + TDZ 0.5 mg/l + NAA 1.0 mg/l	2/4 (50.0)	5/6 (83.3)	3/6 (50.0)	6/13 (46.2)	8/10 (80)	0/5 (0)
E5	MS + 3% sucrose + TDZ 1.0 mg/l	2/9 (22.2)	0/19 (0)	0/10 (0)	1/9 (11.1)	13/24 (54.2)	3/15 (20.0)

Percent values are indicated within brackets

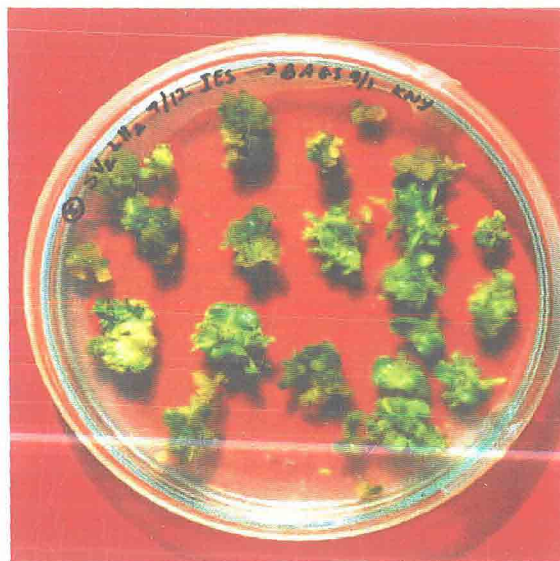


Plate 4.5 Immature embryos cultured on SV2 medium showing formation of multiple shoots

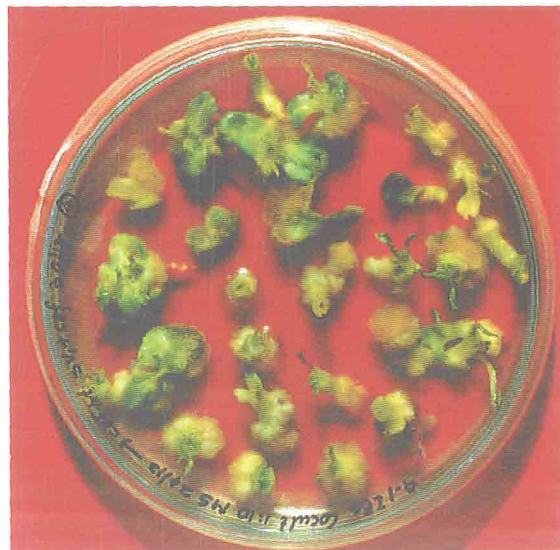


Plate 4.6 Immature embryo axes transformed by *Agrobacterium* mediated method and cultured on SV1 medium



Plate 4.7 Immature cotyledons subjected to *Agrobacterium* infection and cultured on SV1 medium

continuous supply of material and less laborious explant preparation, compared to using immature embryos as explants for genetic transformation of safflower, for further transformation studies in this investigation, seedling explants in conjunction with the established in house protocols were utilized.

4.3 Cloning of the identified mitochondrial gene(s) in appropriate orientation to develop the constructs.

Substantial editing observed in *nad3* and *atp9* transcripts in safflower indicated their suitability as candidates for induction of transgenic male sterility. Hence constructs for induction of male sterility and corresponding constructs for fertility restoration using these genes were developed.

4.3.1 Development of constructs for induction of male sterility

4.3.1.1 Cloning of *nad3* and *atp9* genes in T/A vector

nad3 and *atp9* genes amplified from safflower mitochondrial DNA (using FII and RII primers which amplified the coding region of the gene), were eluted and ligated into T/A cloning vector to develop the genomic clones. Cloning was confirmed by restriction with *HindIII* and *EcoRI* (Figure 4.15). Orientation of the inserts was confirmed by restriction with *SacI* enzyme. Since *SacI* site was included in the reverse primer, and one site is present in the MCS of vector upstream of the cloning site, release of a fragment (approx. 370 bp for *nad3* and 270bp for *atp9*) by *SacI* restriction indicates the cloning of insert in the sense orientation whereas no visible fragment indicated the cloning of the insert in the antisense orientation. The former orientation clones are not desirable, since they give the expected size

fragment with single enzyme digestion. Since it is intended to clone the fragment in correct orientation using *KpnI* and *SacI* enzymes, cloning by mistake, with a single enzyme (only *SacI* or only *KpnI*) would complicate/restrict the downstream operations. Hence confirmation of orientation of all T/A clones is very important and was followed through out this investigation. One confirmed clone for each of these genes (genomic-unedited) was sequenced to assess whether any PCR induced mutations were included in the clones. It was observed that the selected clones were free of such mutations and hence were used for development of constructs in subsequent experiments.

4.3.1.2 Development of pCAMBIA1305.2-TCNN and pCAMBIA1305.2-TCAN

Earlier in the biotechnology laboratory, gene construct for induction of male sterility had been developed using *orfH522* gene from sunflower (Harinath, 2004). The gene cassette TCON had the following components in the 5' to 3' orientation: TA29 (tapetum specific promoter from tobacco), *coxIV* (presequence from yeast), *orfH522* (from sunflower) and *nos* terminator. This gene cassette had been cloned in the binary vector pCAMBIA1305.2. In the present study, this vector was used as basal vector for cloning the unedited *nad3* and *atp9* genes. The confirmed T/A-*nad3* and T/A-*atp9* genomic clones were restricted with *KpnI* and *SacI* enzymes to release the required gene fragments (363 bp for *nad3* and 267 bp for *atp9*). Simultaneously, a confirmed clone of pCAMBIA1305.2-TCON (TA29 promoter, *coxIV* transit peptide, *orfH522* and *nos* terminator) was cut with the same enzymes so that the *orfH522* fragment (531bp) could be excised out from the gene cassette. The vector band and the insert gene fragments (*nad3* and *atp9*) were gel eluted and ligated to develop the constructs pCAMBIA 1305.2-TCNN (TA29 promoter, *coxIV* transit peptide, unedited

version of *nad3* and *nos* terminator) and pCAMBIA1305.2-TCAN (TA29 promoter, *coxIV* transit peptide, unedited version of *atp9* and *nos* terminator). Six colonies each of pCAMBIA1305.2-TCNN and pCAMBIA1305.2-TCAN were subjected to colony PCR for *nad3* and *atp9* genes. Plasmids of five clones each, which gave the expected amplicons in colony PCR, were isolated. All these clones were confirmed by restriction with *KpnI* and *SacI* enzymes that released the cloned gene fragments. Three clones each were used to set up serial PCR to confirm the presence and orientation of all the four component sequences of the gene cassette i.e., TA29 promoter, *coxIV* transit peptide, *nad3/atp9* genes and *nos* terminator (Figure 4.31a & b). The forward primer 'SphI TA29 For' was same for all the four combinations whereas the reverse primer for all the combinations were different and corresponded to reverse primers of each of the four component sequences. PCR with all the four combinations of primers yielded the expected size amplicons in all these pCAMBIA1305.2-TCNN and pCAMBIA1305.2-TCAN clones confirming the proper orientation of both the cassettes (Figure 4.31a & b).

4.3.1.3 Cloning of TCNN and TCAN into pCAMBIA0390

Binary vector pCAMBIA1305.2 has hygromycin resistance as selection agent and this cannot be used in the field easily (for killing/removing fertile plants during maintenance of the transgenic male sterile plants). Therefore, *bar* gene cassette that confers resistance to herbicide phosphinothricin (or Basta) had to be cloned in the vector. As a preliminary step, the male sterility induction gene cassette had to be cloned into pCAMBIA0390 vector that does not carry any plant selection marker so that subsequently *bar* gene cassette could be cloned into the resultant binary vector. For this purpose pCAMBIA1305.2-TCNN and

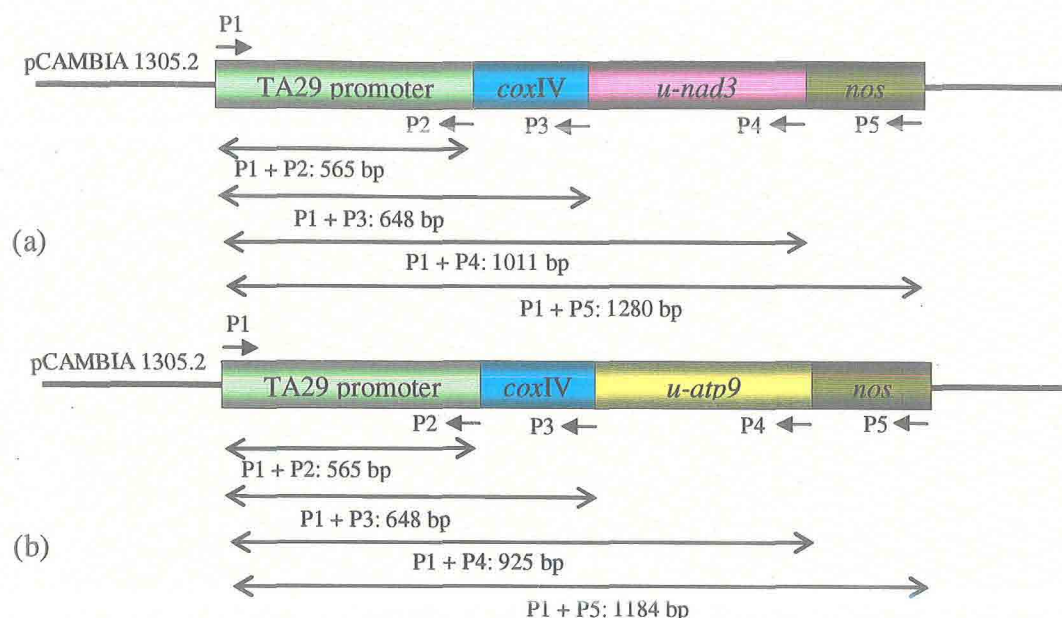


Fig. 4.31a Schematic diagram of primers used for serial PCR and the amplicons obtained for pCambia1305.2-TCNN and pCambia1305.2-TCAN

(a) 'TCNN' clone	P1- <i>SphI</i> TA29 F primer	P2- <i>SalI</i> TA29 R primer
P3- <i>KpnI</i> <i>coxIV</i> R primer	P4- <i>SacI</i> <i>nad3</i> R2' primer	P5- <i>EcoRI</i> <i>nos</i> R primer
(b) 'TCAN' clone	P1- <i>SphI</i> TA29 F primer	P2- <i>SalI</i> TA29 R primer
P3- <i>KpnI</i> <i>coxIV</i> R primer	P4- <i>SacI</i> <i>atp9</i> R2' primer	P5- <i>EcoRI</i> <i>nos</i> R primer



Fig. 4.31b Serial PCR for confirmation of pCambia1305.2-TCNN and pCambia1305.2-TCAN

Lanes 1, 5&9. P1+P2; **2, 6&10.** P1+P3; **3,7&11.** P1+P4 *nad3*R (upper panel); **3,7&11.** P1+P4 *atp9*R (lower panel); **4,8&12.** P1+P5; **M₁.** λ (*HindIII*+*EcoRI*) marker; **M₂.** 100bp ladder; **13.** pCambia1305.2-TCNN with P1+P2 (UP); **14.** pCambia1305.2-TCNN with P1+P3 (UP); **13.** pCambia1305.2-TCAN with P1+*nad3*R+*atp9*R (LP); **14.** pCambia1305.2-TCAN with P1+P5 (LP).

pCAMBIA1305.2-TCAN vectors were restricted with *HindIII* and *EcoRI* enzymes to excise out the TCNN (1.38kb) and TCAN (1.18kb) gene cassettes. pCAMBIA0390 vector (6.8kb) was also restricted with the same enzymes and used for ligation with the isolated gene cassettes to generate pCAMBIA0390-TCNN and pCAMBIA0390-TCAN. Twelve clones each of both the vectors were subjected to colony PCR for *nad3/atp9* genes. Six clones of pCAMBIA0390-TCNN and five clones of pCAMBIA0390-TCAN, which gave the expected amplicons during colony PCR, were selected. These 11 clones were confirmed by restriction with *HindIII* and *EcoRI*. Presence of a 1.38 kb fragment for TCNN and a 1.18 kb for TCAN in the restriction profiles confirmed the presence of gene cassettes in these vectors (Figure 4.32).

4.3.1.4 Cloning of the bar gene cassette into pCAMBIA0390-TCNN and pCAMBIA0390-TCAN

Phosphinothricin resistance gene cassette was available in pBARGUS vector available in the laboratory. In this vector '*bar*' gene is under CaMV35S promoter with *nos* terminator as the transcription terminator. The entire '*bar*' gene cassette could be taken out as a *HindIII* fragment from pBARGUS. Several attempts to clone this bar gene cassette using *HindIII* into pCAMBIA0390-TCON, pCAMBIA0390-TCNN and pCAMBIA0390-TCAN failed. Hence an attempt was made to first clone the cassette into a smaller vector pUC18 and subsequently clone it into the required binary vector.

The *bar* gene cassette (2.1kb) was taken out from pBARGUS (9.2 kb) using *HindIII* digestion and ligated into *HindIII* digested pUC18 vector (2.68 kb). One positive clone (pUC18-Bar clone 6) out of the six clones tested, showed a 2.1kb fragment upon restriction

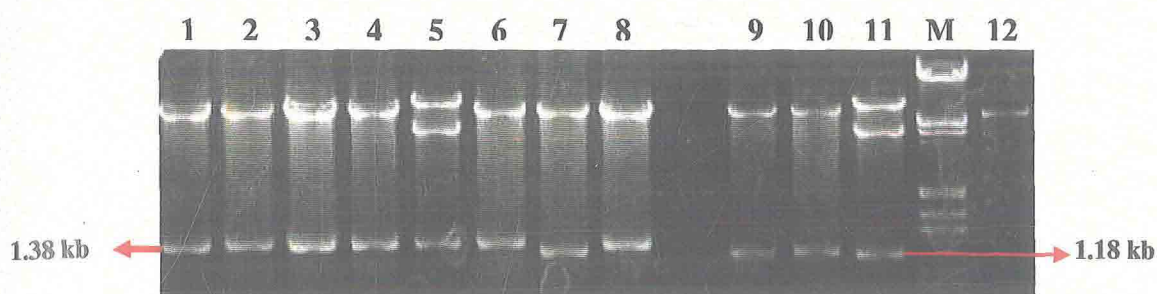


Fig. 4.32 Confirmation of pCAMBIA0390-TCNN and pCAMBIA0390-TCAN by restriction with *Hind*III and *Eco*RI

Lanes 1 to 4. pCAMBIA0390-TCNN clones; 6 to 10. pCAMBIA0390-TCAN clones; 5. pCAMBIA1305.2-TCNN (positive control); 11. pCAMBIA1305.2-TCAN (positive control); 12. pCAMBIA0390; M. λ (*Hind*III + *Eco*RI) marker.

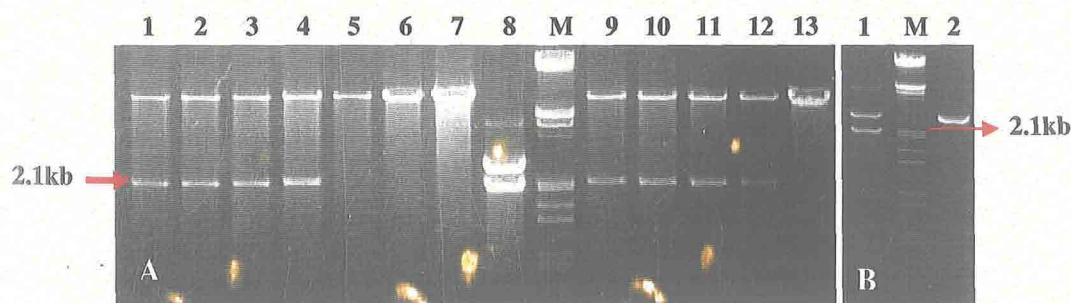


Fig. 4.33 Confirmation of pBin-TCNN-Bar and pBin-TCAN-Bar

A) Restriction of pBin-TCNN-Bar and pBin-TCAN-Bar clones with *Hind*III

Lanes 1 to 4. positive pBin-TCNN-Bar clones; 5 and 6. negative pBin-TCNN-Bar clones; 7. pCAMBIA0390-TCNN (negative control); 9 to 12. positive pBin-TCAN-Bar clones; Lanes 13. pCAMBIA0390-TCAN (negative control); 8. pUC18-Bar; M. λ (*Hind*III+*Eco*RI) marker

B) Restriction of pUC18-Bar clone with *Hind*III; 1. pUC18-Bar clone; 2. pUC18 (control).

with *HindIII* (Figure 4.33b). This positive clone along with pCAMBIA0390-TCON, pCAMBIA0390-TCNN and pCAMBIA0390-TCAN were all restricted with *HindIII* and ligations were set up to generate pBin-TCON-Bar, pBin-TCNN-Bar and pBin-TCAN-Bar. Restriction analysis with *HindIII* and *EcoRI* confirmed that out of the six pBin-TCNN-Bar clones, four were positive (had the 2.1 kb bar construct) whereas all the four pBin-TCAN-Bar clones were positive (Figure 4.33a).

Orientation of the bar gene cassette with respect to the TCNN/TCAN was confirmed by the restriction of these clones with *BamHI* and *SacI*. The *SacI* enzyme was one of the cloning enzymes used in the male sterility causing constructs whereas the *BamHI* site lies within the *bar* cassette in such a way that restricted fragment with these two sites would give a 50 bp difference depending on the orientation of the *bar* cassette (Figure 4.34a & 4.34b). Of the four positive pBin-TCNN-Bar clones, two had the *bar* gene cassette in the same orientation as the TCNN construct and upstream of it whereas it was in the opposite orientation in the other two clones. But all the four positive pBin-TCAN-Bar clones had the *bar* cassette cloned in the opposite orientation with respect to the TCAN construct. Such clones which had the *bar* cassette in the opposite orientation compared to the male sterility inducing construct were chosen and used for further investigations.

4.3.2 Development of constructs for restoration of fertility

Fertility restoration in the transgenic male sterile lines could be achieved by silencing the gene that induces male sterility. Silencing of genes has been reported by different PTGS technologies in several crops. Among these approaches, a classical approach is the antisense approach and the recent and more efficient approach is the production of

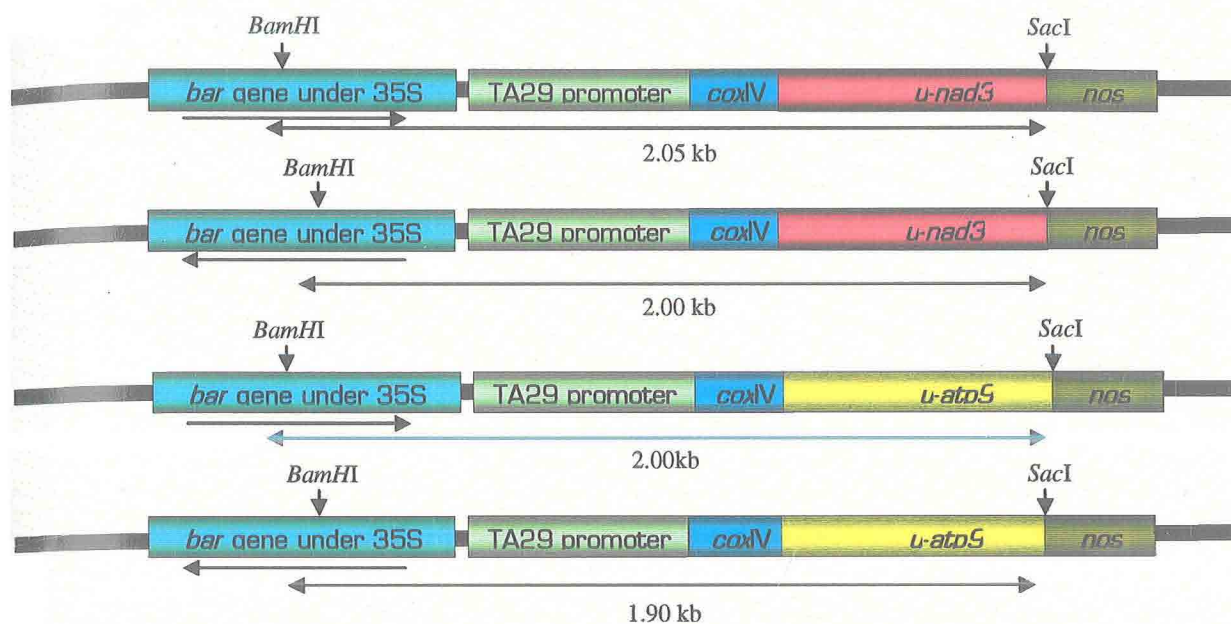


Fig. 4.34a Schematic representation for confirmation of orientation of bar cassette vs. male sterility gene cassette by restriction analysis

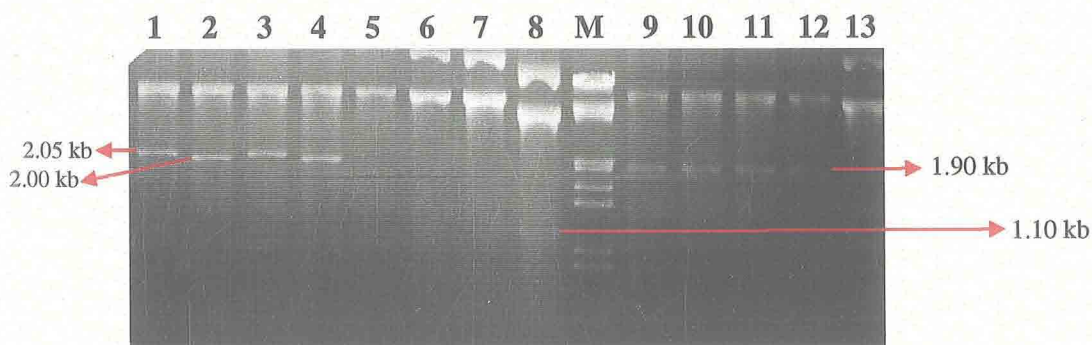


Fig. 4.34b Confirmation of orientation of pBin-TCNN-Bar and pBin-TCAN-Bar Restriction with *Bam*HI and *Sac*I

Lanes 1 to 4. positive pBin-TCNN-Bar clones; 5 and 6. negative pBin-TCNN-Bar clones; 7. pCambia0390-TCNN (negative control); 9 to 12. positive pBin-TCAN-Bar clones; 13. pCambia0390-TCAN (negative control); 8. pUC18-Bar; M. λ (HindIII + EcoRI) marker.

intron-interrupted hairpin RNA (ihp-RNA) against target mRNA. For the male sterility inducing genes, *u-nad3* and *u-atp9*, full-length antisense as well as ihp-RNA constructs were developed. The results obtained at various steps are presented here.

4.3.2.1 Development of full-length antisense *nad3* and *atp9* constructs under CaMV 35S promoter

pRT100 vector which has CaMV 35S promoter as well as polyA terminator spaced by MCS was used as backbone to clone the genes in antisense orientation. The advantage of this vector is that once the gene/gene fragment is cloned between the promoter and the terminator, the entire gene cassette could be taken out using *HindIII/PstI* enzyme.

4.3.2.1.1 Cloning full-length *nad3* and *atp9* genes in pRT100 in the antisense orientation

nad3 and *atp9* genes were cloned in antisense orientation by using *KpnI* and *SacI* restriction enzymes which would ensure the required orientation of the genes in the constructs. The same T/A-*nad3* and *atp9* positive clones, which were used during development of the male sterility constructs, were used. pRT100 vector as well as the T/A clones were restricted with *KpnI* and *SacI*. The linearized vector was ligated to these two gene fragments.

The clones were confirmed by restriction with *KpnI* and *XhoI* (Figure 4.35a & b). Since *XhoI* site was present upstream of *SacI* site, a band of approximately 380 bp for *nad3* and 280 bp band for *atp9*, not only confirmed the presence of the insert but also the antisense orientation of inserts in all the clones. The clones were designated as p-SANP

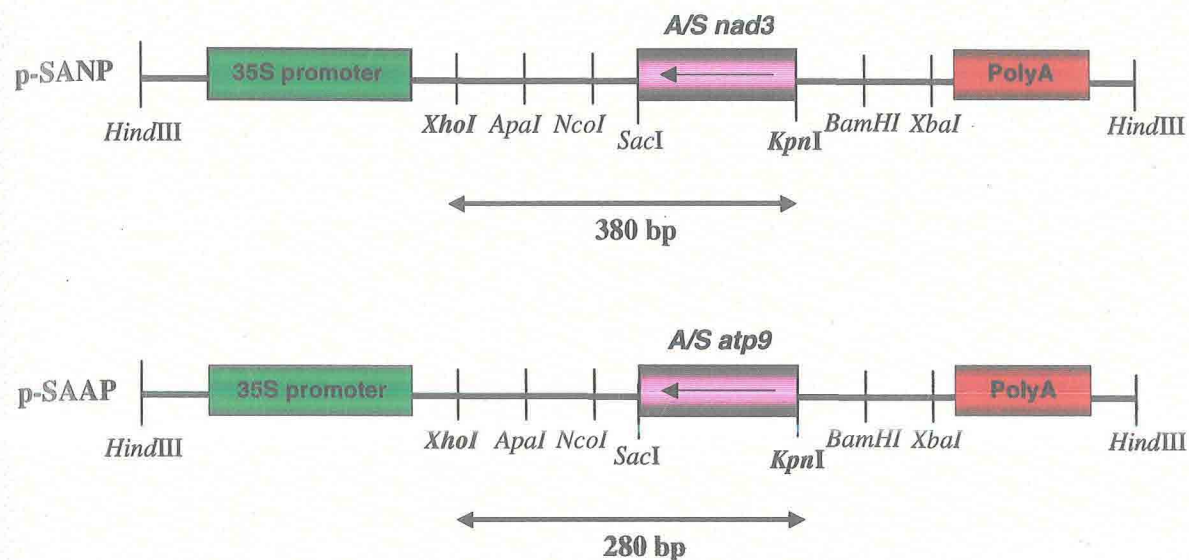


Fig. 4.35a Schematic representation of confirmation of p-SANP and p-SAAP by restriction with *KpnI* and *XhoI*

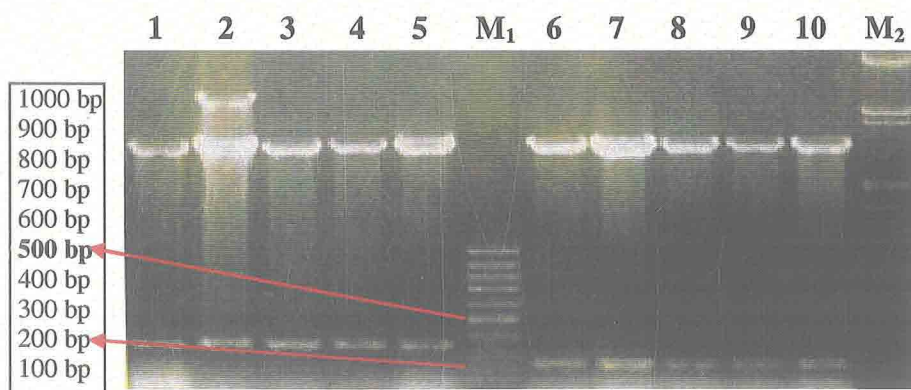


Fig. 4.35b Confirmation of p-SANP and p-SAAP by restriction with *KpnI* and *XhoI*

Lanes 1 to 5. pRT100-SANP clones; 6 to 10. pRT100-SAAP clones;
M₁. 100bp ladder; M₂. λ (HindIII + EcoRI) marker

(CaMV35S promoter-full length antisense-*nad3* gene-polyA terminator) and p-SAAP (CaMV 35S promoter-full length antisense-*atp9* gene-polyA terminator).

4.3.2.1.2 Cloning of the SANP and SAAP constructs into pCAMBIA 1300

The SANP and SAAP cassettes were excised out (from p-SANP and p-SAAP) using *HindIII* enzyme (Figure 4.36) and cloned into pCAMBIA1300 (restricted with *HindIII*) and ligated to generate pBin-SANP and pBin-SAAP clones. These clones were confirmed by restriction with *HindIII* (by the release of the inserts; 1.1kb for SANP and 0.96 kb for SAAP) (Figure 4.37).

4.3.2.2 Development of Intron Hairpin (ihp) RNA constructs using *nad3* and *atp9* under CaMV 35S promoter

Since literature revealed that ihp-RNA constructs targeted against a particular gene transcript, are more effective in gene silencing than full-length antisense constructs, such constructs were developed for restoration of fertility. For this purpose, the strategy adopted involved, cloning the *nad3/atp9* genes in antisense and sense orientation upstream and downstream of a functional catalase intron. In the biotechnology laboratory, already generic vectors for developing constructs that yield ihp-RNA against target genes were available. In this generic vector, a functional catalase intron has been cloned in the MCS of pRT100 vector, between 35S promoter and polyA sequence (pRT100-CI) (Figure 3.8) so that the required gene/fragment of the target gene could be cloned upstream and downstream of the catalase intron. To achieve this *u-nad3* and *u-atp9* genes were amplified using a new set of

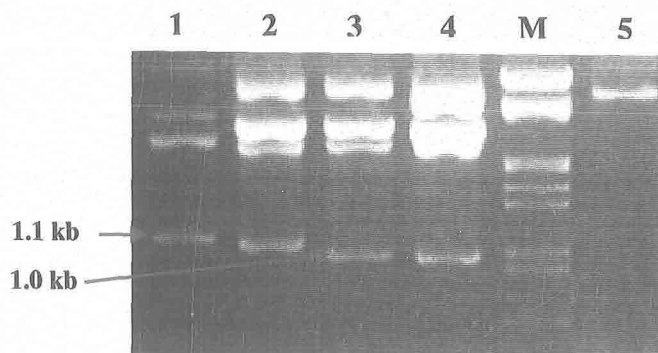


Fig. 4.36 Restriction of p-SANP and p-SAAP with *Hind*III

Lanes 1 and 2. p-SANP clones; 3 and 4. p-SAAP clones; 5. pCAMBIA1300; M. λ (*Hind*III + *Eco*RI) marker.



Fig. 4.37 Confirmation of pBin-SANP and pBin-SAAP by restriction with *Hind*III

Lanes 1 to 3. pBin-SANP clones; 4. p-SANP (positive control); 5 to 8. pBin-SAAP clones; 9. p-SAAP (positive control); 10. pCAMBIA1300 (negative control); 11. uncut pBin-SANP; 12. uncut pBin-SAAP; M2. λ (*Hind*III + *Eco*RI) marker.

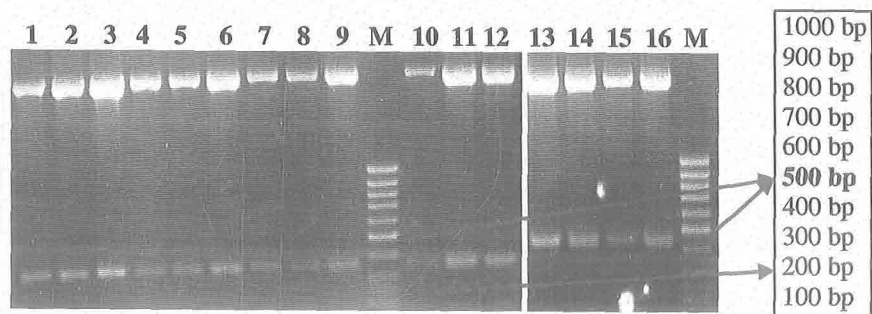


Fig. 4.38 Confirmation of T/A-*nad3* (ihp) and T/A-*atp9* (ihp) by restriction with *Hind*III and *Eco*RI

Lanes 1 to 12. T/A-*atp9* (ihp) clones; 13 to 16. T/A-*nad3* (ihp); M. 100 bp ladder.

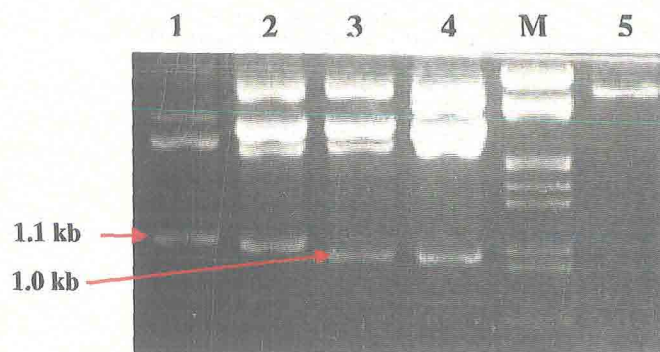


Fig. 4.36 Restriction of p-SANP and p-SAAP with *Hind*III

Lanes 1 and 2. p-SANP clones; 3 and 4. p-SAAP clones; 5. pCAMBIA1300; M. λ (*Hind*III + *Eco*RI) marker.



Fig. 4.37 Confirmation of pBin-SANP and pBin-SAAP by restriction with *Hind*III

Lanes 1 to 3. pBin-SANP clones; 4. p-SANP (positive control); 5 to 8. pBin-SAAP clones; 9. p-SAAP (positive control); 10. pCAMBIA1300 (negative control); 11. uncut pBin-SANP; 12. uncut pBin-SAAP; M2. λ (*Hind*III + *Eco*RI) marker.

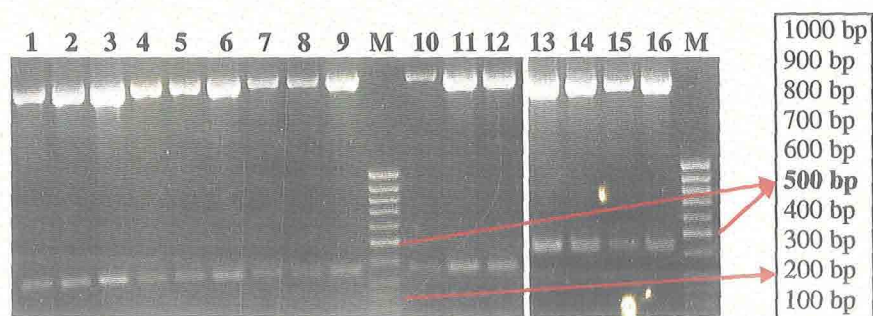


Fig. 4.38 Confirmation of T/A-*nad3* (ihp) and T/A-*atp9* (ihp) by restriction with *Hind*III and *Eco*RI

Lanes 1 to 12. T/A-*atp9* (ihp) clones; 13 to 16. T/A-*nad3* (ihp); M. 100 bp ladder.

primers (Table 3.8) in which appropriate restriction enzyme sites were included to facilitate the easy cloning of the genes in both sense and antisense orientations.

4.3.2.2.1 Cloning of unedited versions of *nad3* and *atp9* in T/A vector

From the earlier confirmed T/A clones (which were used for development of constructs for induction of male sterility), *u-nad3* and *u-atp9* genes were amplified using the new sets of primers. The amplified fragments were cloned into T/A vector to develop T/A-*nad3* (ihp) and T/A-*atp9* (ihp) clones. Presence of the insert was confirmed by restriction with *HindIII* and *EcoRI* that gave 432 bp band for *nad3* and 336 bp band for *atp9* (Figure 4.38). The orientation of the insert in the clones was confirmed by restriction with *BamHI*. A 385 bp fragment in *nad3* clones and 289 bp fragment in the *atp9* clones indicated the antisense (*BamHIXhoI-SacIKpnI*) orientation whereas no visible fragment (as the band was expected to be only 10 bp) indicated the sense (*KpnISacI-XhoIBamHI*) orientation of the insert in the T/A vector (Figure 4.39a & b).

4.3.2.2.2 Cloning of *nad3* and *atp9* genes in the antisense orientation into pRT100-catalase intron (pRT-CI)

One each of confirmed clones of T/A-*nad3* (ihp) and T/A-*atp9* (ihp) were restricted with *SacI* and *XhoI* enzymes. The excised fragments were cloned into p-CI vector also restricted with *SacI* and *XhoI* enzymes. The choice of these enzymes facilitated the cloning of the insert in the antisense orientation upstream of the catalase intron. The clones were confirmed by restriction with *HindIII*, which gave a 1.4 kb for p-CI-Antisense *nad3* (pRT100 vector with catalase intron and antisense *nad3*) clones and 1.3 kb for pRT100-CI-Antisense *atp9* (pRT100 vector with catalase intron and antisense *atp9*) clones. Two among

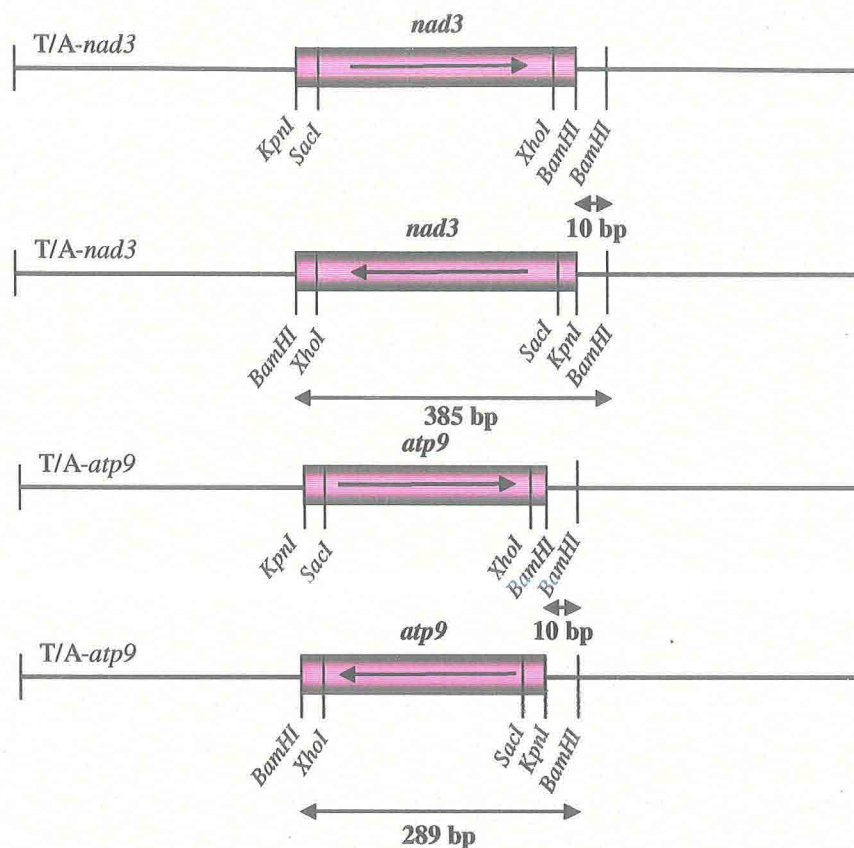


Fig. 4.39a Confirmation of the orientation of T/A-*nad3* (ihp) and T/A-*atp9* (ihp) by restriction with *Bam*HI



Fig. 4.39b Confirmation of the orientation of T/A-*nad3* (ihp) and T/A-*atp9* (ihp) by restriction with *Bam*HI

Upper panel: Lanes 1 to 12. T/A-*nad3* (ihp) clones (no DNA in lanes 3 to 9); 14 & 15. uncut T/A-*nad3* (ihp) clones; 13. T/A *nad3* clone (developed earlier for TCNN construct).

Lower panel: Lanes 1 to 12. T/A-*atp9* (ihp) clones; 14 & 15. uncut T/A-*atp9* (ihp) clones; 13. T/A-*atp9* clone (developed earlier for TCAN construct); M. 100 bp ladder.

the p-CI-Antisense *nad3* clones did not carry the insert whereas all the p-CI-Antisense *atp9* clones had the insert (Figure 4.40). The negative clones indicated the re-ligated vector sequences, perhaps because of partial digestion of the vector DNA with either of the enzymes used for cloning. During this restriction, partial digestion of the plasmid DNA was seen because of higher concentration of the DNA (Figure 4.40). But this did not alter the interpretations as only the released fragment (seen clearly for all the clones) is the indicator of cloning.

4.3.2.2.3 Cloning of *ihp nad3* and *ihp atp9* genes in the sense orientation into p-CI-Antisense *nad3* and p-CI-Antisense *atp9*

T/A-*nad3* (*ihp*) and pRT100-CI-Antisense *nad3* were restricted with *Bam*HI and *Kpn*I and the *u-nad3* insert was ligated to the vector (pRT100-CI-Antisense *nad3*) to generate p-*ihp nad3* clones. In a similar way p-*ihp atp9* clones were also generated. These clones were confirmed by restriction analysis with *Hind*III enzyme. Of the nine p-*ihp nad3* clones, six were positive showing the 1.6 kb fragment whereas three clones had the 1.4 kb insert indicating the re-ligation of the recipient vector band (Figure 4.41) while among the ten p-*ihp atp9* clones, only three were positive yielding the 1.5 kb fragment when restricted (Figure 4.42).

4.3.2.2.4 Cloning of *ihp nad3* and *ihp atp9* constructs into pCAMBIA1300

From the positive vectors, the entire cassettes i.e., CaMV 35S promoter-*ihp nad3*-polyA terminator and CaMV 35S promoter-*ihp atp9*-polyA terminator, were excised out using *Hind*III enzyme and were cloned into *Hind*III digested pCAMBIA1300. These clones

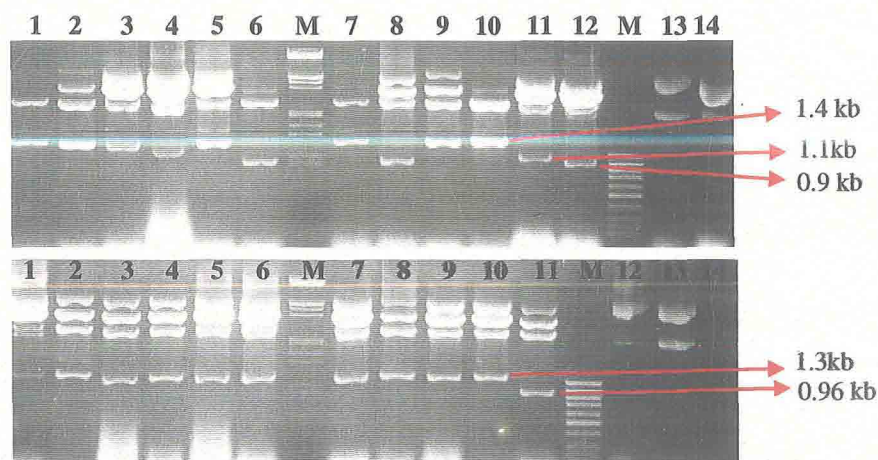


Fig. 4.40 Confirmation of pRT-CI-Antisense *nad3* and 1.3 kb for pRT-CI-Antisense *atp9* by restriction with *Hind*III

Upper panel: Lanes 1 to 10. pRT100-CI-Antisense *nad3* clones (Lanes 6&8 indicate clones without insert); 11. p-SANP (positive control); 12. pRT100-CI (negative control); 13&14. uncut pRT100-CI-Antisense *nad3* clones.

Lower panel: Lanes 1 to 10. pRT100-CI-Antisense *atp9* clones; 11. p-SAAP (positive control); 12&13. uncut pRT100-CI-Antisense *atp9* clones.

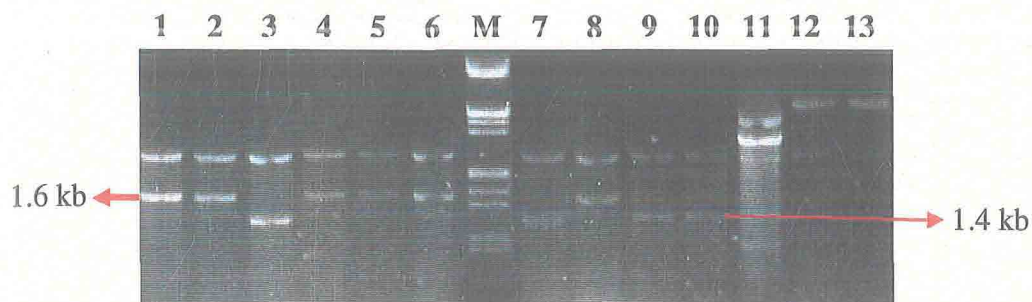


Fig. 4.41 Confirmation of p-ihp *nad3* clones by restriction with *Hind*III

Lanes 1 to 9, p-ihp *nad3* clones; 10, pRT100-CI-Antisense *nad3*; 12&13. uncut p-ihp *nad3* clones; 11, pRT100-CI; M. λ (*Hind*III + *Eco*RI) marker.

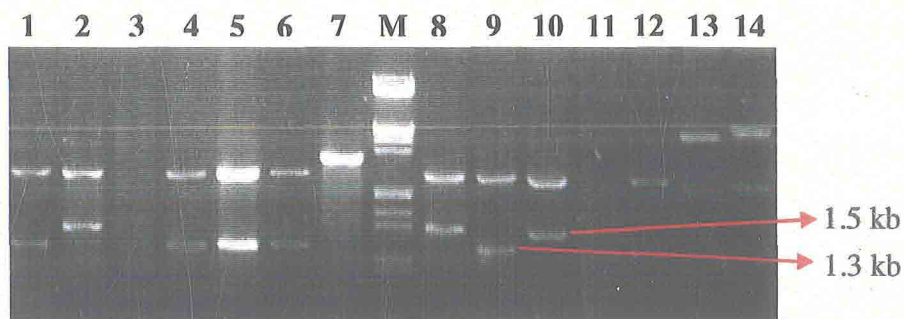


Fig. 4.42 Confirmation of p-ihp *atp9* clones by restriction with *Hind*III

Lanes 1 to 10, p-ihp *atp9* clones; 12, pRT100-CI-Antisense *atp9*; 13&14, uncut pRT100-ihp *atp9* clones; 11, pRT100-CI; M. λ (*Hind*III + *Eco*RI) marker.



Fig. 4.43 Confirmation of pBin-ihp *nad3* and Bin-ihp *atp9* clones by restriction with *Hind*III

Lanes 1 to 5, pBin-ihp *nad3* clones; 8 to 10, pBin-ihp *atp9* clones; 6, p-ihp *nad3*; 11, p-ihp *atp9*; 7, pRT100-CI-Antisense *nad3*; 12, pRT100-CI-Antisense *atp9*; M. λ (*Hind*III + *Eco*RI) marker.

were confirmed by restriction with *HindIII*. Three positive clones of pBin ihp *nad3* yielded the expected 1.6 kb fragment and only one clone of pBin-ihp *atp9* clone gave the expected 1.5 kb band (Figure 4.43). Thus the ihp vectors with *nad3* and *atp9* were constructed.

4.3.2.3 Development of full-length antisense *nad3* and *atp9* constructs under TA29 promoter

Though constructs for restoration of fertility were developed under a constitutive 35S promoter, it was later intended to replace the 35S promoter by tapetal specific TA29 promoter. This was because 35S promoter is known to have low expression in floral organs and more so in tapetum cells. Since constructs for induction of male sterility were under TA29 promoter, the restoration constructs too under the same promoter would increase the efficiency of gene silencing, thereby leading to more efficient restoration of fertility.

4.3.2.3.1 Cloning of TA29 by replacing CaMV 35S promoter in pSANP and pSAAP

One each of the confirmed p-SANP and p-SAAP (which had CaMV 35S promoter) clones were restricted with *HincII* and *XhoI* enzymes that released the CaMV 35S promoter from the vector. TA29 promoter from T/A-TA29 available in the laboratory was also excised out with *HincII* and *XhoI* enzymes and was cloned into the restricted p-SANP and p-SAAP. The developed pTANP (TA29 promoter-full length antisense-*nad3* gene-polyA terminator) and pTAAP (TA29 promoter-full-length antisense-*atp9* gene-polyA terminator) clones were confirmed by colony PCR. 12 clones each of pTANP and pTAAP were tested, of which 11 and 10 respectively were positive showing the expected amplicons (Figure 4.44). From the colony PCR positive clones, four each of pTANP and pTAAP were used for



Fig. 4.44 Colony PCR for confirmation of presence of TA29 in p-TANP and p-TAAP

PCR with TAF and TAR primers: Lanes 1 to 12. pTANP colonies; 15 to 26. pTAAP colonies; 13 & 27. blanks; 14 & 28. T/A-TA29 DNA (positive control) M. 100bp DNA ladder.

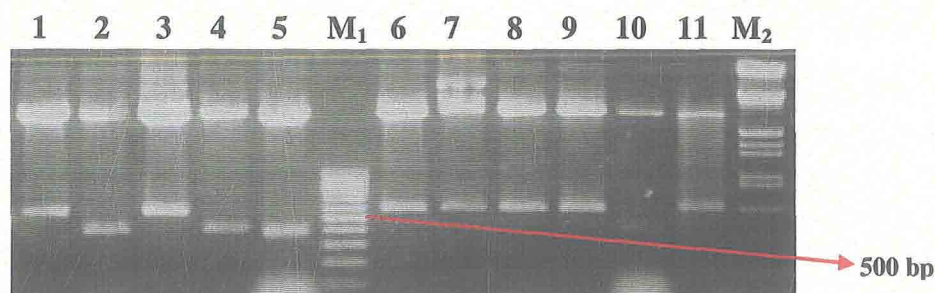


Fig. 4.45 Confirmation of presence of TA29 in pTANP and pTAAP by restriction with *HincII* and *XhoI*

Lanes 1 to 4. p-TANP clones; 6 to 9. p-TAAP clones; 5. p-SANP; 10. p-SAAP; 11. T/A-TA29 (positive control); M2. λ (*HindIII* + *EcoRI*) marker; M1. 100bp DNA ladder.



Fig. 4.46 Colony PCR for confirmation of p-TA29 clones

PCR with TAF and TAR primers; Lanes 1 to 10. p-TA29 colonies; 11. gel eluted TA29; 12. T/A-TA29 (positive control); 13. pRT100 (negative control); 14. gel eluted pRT 100 vector; M. 100bp DNA ladder.

restriction with *HincII* and *XhoI* enzymes. Only two of the pTANP clones, showed the expected 531 bp fragment, where as the remaining two clones showed around 410 bp fragment (similar to the cut pSANP fragment) indicating that in these, the 35S promoter has not been replaced by the TA29 promoter. But all the four pTAAP clones restricted, showed the presence of TA29 promoter (Figure 4.45). Thus the pTANP and pTAAP clones were developed.

4.3.2.3.2 Cloning of the entire TAAP and TANP cassettes into pCAMBIA1300

One each of the confirmed pTANP and pTAAP and also pCAMBIA 1300 were restricted with *HindIII* to obtain the gene cassettes and the recipient vector bands. The fragments were ligated to generate pBin-TANP and pBin-TAAP. The clones were confirmed by restriction with *HindIII*.

4.3.2.4 Development of Intron Hairpin (IHP) RNA constructs using *nad3* and *atp9* under TA29 promoter

4.3.2.4.1 Cloning of TA29 promoter into pRT 100 vector

pRT100 was restricted with *HincII* and *XhoI* enzymes which separated out the 35S promoter from the vector. T/A-TA29 was also restricted with the same enzymes to take out the TA29 promoter fragment (as these enzyme site were present in the TA29 primers). The vector from the former and insert from the latter, were ligated, to generate pRT100-TA29. The clones were confirmed by the presence of approximately 545 bp band after colony PCR (Figure 4.46). Final confirmation by restriction analysis with *HincII* and *XhoI* Enzymes revealed that all the five clones had the expected insert (Figure 4.47).



Fig. 4.47 Confirmation of p-TA29 clones by restriction with *HincII* and *XhoI*

Lanes 1 to 5. pTA29 clones; 6. T/A-TA29 (positive control); 7. pRT100; M₁. 100bp DNA ladder; M₂. λ (*HindIII* + *EcoRI*) marker; 10 and 11. uncut p-TA29 clones; 8 and 9. p-TA29 clones restricted with *XhoI* alone.



Fig. 4.48 Confirmation of p-T-ihp-*nad3* and p-T-ihp-*atp9* clones by restriction with *XhoI*

Lanes 1 to 8. p-T-ihp-*atp9* clones; 9. p-ihp-*atp9* (positive control); 10. pRT100 (negative control); 11&12. p-T-ihp-*nad3* clone; 13. pRT100 (negative control); M₁. 100bp DNA ladder; M₂. λ (*HindIII* + *EcoRI*) marker.

4.3.2.4.2 Cloning of *ihp nad3* and *ihp atp9* into pRT100-TA29

One clone each of p-*ihp nad3* and p-*ihp atp9* were restricted with *XhoI*, which separated out the *ihp nad3* (940 bp) and *ihp atp9* (742 bp) from the vector backbone. These two inserts were ligated to pTA29 vector (linearized by restriction with *XhoI*) to generate p-T-*ihp nad3* and p-T-*ihp atp9*. The developed clones were confirmed by restriction with *XhoI* (940 bp fragment for *ihp nad3* and 742 bp fragment for *ihp atp9*) (Figure 4.48). Confirmation was also done by PCR analysis using primer combinations in such a way that presence of all the components of the gene constructs were confirmed. Expected amplicons with TA29 F and *nad3/atp9* F confirmed the presence of genes in the antisense orientation whereas TA29 F and Cat Int F confirmed the presence of antisense gene along with catalase intron in the construct. Similarly expected amplicons were obtained with Cat Int F and *nad3/atp9* R that indicated the presence of the genes in the sense orientation downstream of catalase intron (Figure 4.49a & b).

4.3.2.4.3 Cloning of the T-*ihp nad3* and T-*ihp atp9* constructs into pCAMBIA1300

A positive clone each of p-T-*ihp nad3*, p-T-*ihp atp9* were restricted with *PstI* and the excised fragments ligated in to pCAMBIA1300 that was also restricted with *PstI* to generate pBin-T-*ihp nad3* and pBin-T-*ihp atp9*. The clones were confirmed by colony PCR (using TA29 Forward and Cat Int Reverse primers) (Figure 4.50). Plasmid DNA were isolated and restriction with *PstI* gave a 1.7 kb fragment for the pBin-T-*ihp nad3* clone and a 1.5 kb fragment for all the five positive pBin-T-*ihp atp9* clones confirming the clones (Figure 4.51). Further PCR with all primer combinations, as mentioned in 4.3.2.4.2, were done to confirm the components of the gene cassettes in these clones (Figure 4.52a & b).

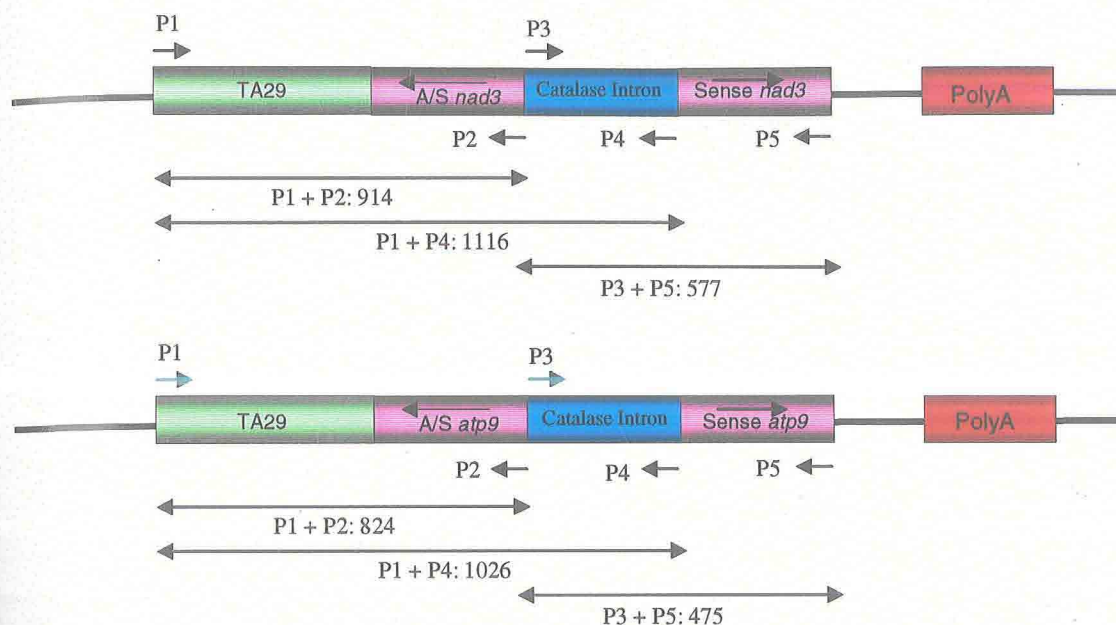


Fig. 4.49a Schematic diagram showing the location of different primers used for the confirmation of p-T-ihp-*nad3* and p-T-ihp-*atp9* clones by PCR using different primer combinations

- (a) 'pT-ihp-*nad3* P1 – *Sph*I TA29 F primer P2 – *Kpn*I *Sac*I *nad3* F primer P3 – Cat Int F primer
P4 – Cat Int R primer P5 – *Bam*HI *Xho*I *nad3* R primer
- (b) 'pT-ihp-*atp9* P1 – *Sph*I TA29 F primer P2 – *Kpn*I *Sac*I *atp9* F primer P3 – Cat Int F primer
P4 – Cat Int R primer P5 – *Bam*HI *Xho*I *atp9* R primer

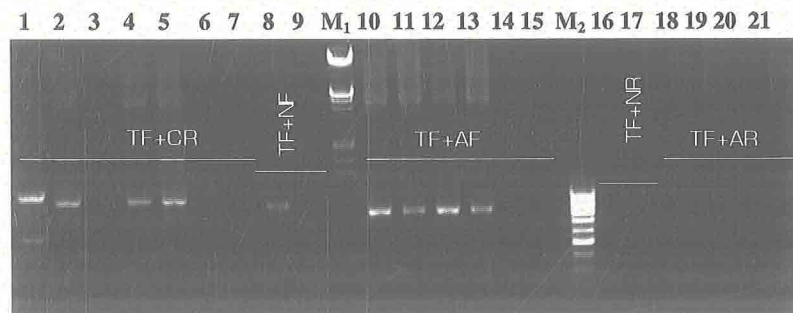


Fig. 4.49b Confirmation of p-T-ihp-*nad3* and p-T-ihp-*atp9* clones by PCR

Lanes 1,8&16. p-T-ihp-*nad3*; 2 to 5, 10 to 14, 18 to 21. p-T-ihp-*atp9* clones; 6, 14. p-ihp-*atp9*; 16&17. p-ihp-*nad3*; 7, 9&15. blanks; M₁. 100bp DNA ladder; M₂. λ (*Hind*III + *Eco*RI) marker.

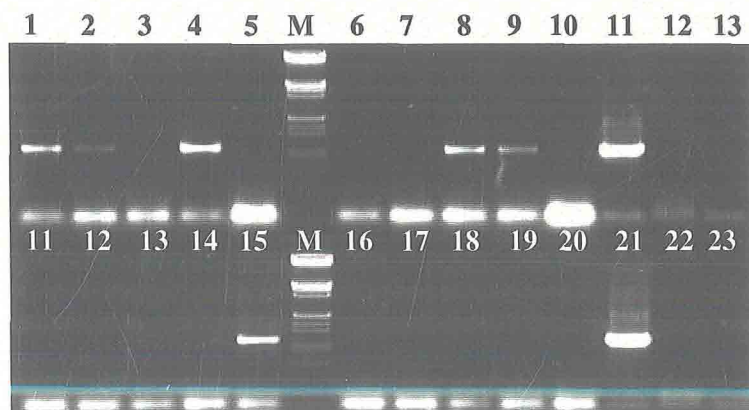


Fig. 4.50 Colony PCR for confirmation of pBin-T-ihp-*nad3* and pBin-T-ihp-*atp9* clones (TAF+CIR primers)

Lanes 1 to 10. pBin-T-ihp-*atp9* clones; 11. p-T-ihp-*atp9* (positive control); 11 to 20. pBin-T-ihp-*nad3* clones; 21. p-T-ihp-*nad3* (positive control); 12&22. pCAMBIA1300 (negative control); 13 & 23. blanks.

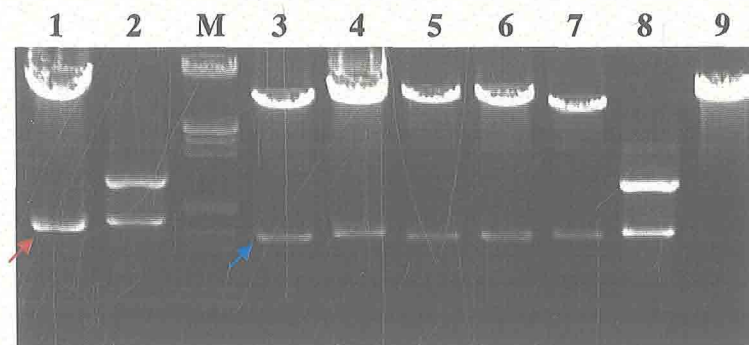


Fig. 4.51 Confirmation of pBin-T-ihp-*nad3* and pBin-T-ihp-*atp9* clones by restriction with *Pst*I

Lane 1. pBin-T-ihp-*nad3* (1.7 kb fragment-red arrow); 2. p-T-ihp-*nad3* (positive control); 3 to 7. pBin-T-ihp-*atp9* clones (1.5kb fragment-blue arrow); 8. p-T-ihp-*atp9* (positive control); 9. pCAMBIA1300 (negative control).

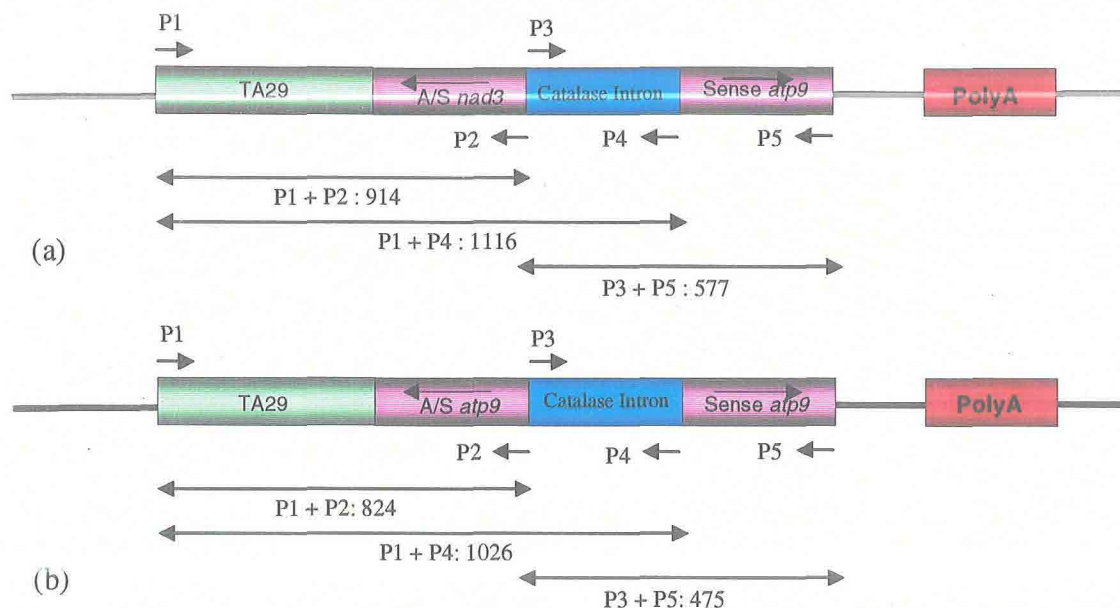


Fig. 4.52a Schematic diagram showing the location of different primers used for confirmation of pBin-T-ihp *nad3* and pBin-T-ihp *atp9* clones by PCR using different primer combinations

- (a) pBin-T-ihp-*nad3* P1 – *Sph*I TA29 F primer P2 – *Kpn*ISacI *nad3* F primer
 P3 – Cat Int F primer P4 – Cat Int R primer P5 – *Bam*HIXhoI *nad3* R primer
 (b) pBin-T-ihp-*atp9* P1 – *Sph*I TA29 F primer P2 – *Kpn*ISacI *atp9* F primer



Fig. 4.52b Confirmation of pBin-T-ihp-*nad3* and pBin-T-ihp-*atp9* clones by PCR

Upper Panel: Lanes 1,4,7,10&13. P1+P2; 2,5,8,11&14. P1+P4; 3,6,9&12. P3+P5; 1-3. pBin-T-ihp *nad3* clone; 4-6. p-T-ihp *nad3* (positive control); 7-9,10-12. pBin-T-ihp *atp9* clones; 13&14. p-T-ihp *atp9* (positive control);
 Lower Panel: Lanes 2&7. P1+*nad3*F (P2); 3&8. P1+*atp9*F (P2); 4&9. P1+P4; 5&10. P3+*nad3*R (P5); 6&11. P3+*atp9*R (P5); 1. p-T ihp *atp9* (positive control); 2-6. pCambia1300 (negative control); 7-11. Blanks (no DNA)
 M₁ 100bp ladder M₂ λ (*Hind*III+*Eco*RI) marker.

Thus all the gene constructs with *atp9* and *nad3* genes for both induction of male sterility and for restoration of fertility were developed and the gene cassettes were cloned into suitable binary vectors. The plasmid maps of these developed recombinant binary vectors are given in appendices I to X. These were to be mobilized into *Agrobacterium* for further use in transformation of tobacco and safflower.

4.4 *Agrobacterium*-mediated transformation of safflower and tobacco with the developed constructs

4.4.1 Mobilization and confirmation of the developed constructs into *Agrobacterium*

All the developed recombinant binary vectors (mentioned above) were mobilized into *Agrobacterium* strain LBA4404 by using the electroporation method. *Agrobacterium* plasmid DNA of the clones were isolated and the mobilizations were confirmed by restriction analysis and/or PCR. Since most of the times, it is difficult to obtain sufficient concentrations of *Agrobacterium* plasmid DNA, another strategy called reverse mating was also adopted for thorough confirmation of the vectors. The binary plasmid DNA from *Agrobacterium* were transferred back into *E. coli* and the presence of the clones in *E. coli* was confirmed using restriction and/or PCR and also by sequencing. The results obtained during these confirmations for each of the recombinant vectors developed are given below.

4.4.1.1 Confirmation of p-Bin-TCNN-Bar and p-Bin-TCAN-Bar in LBA4404

The p-Bin-TCNN-Bar and p-Bin-TCAN-Bar clones in *E. coli* were again confirmed once (Figure 4.53) before mobilizing into *Agrobacterium*. The developed LBA4404: pBin-TCNN-Bar and LBA4404: p-Bin-TCAN-Bar clones were confirmed by restriction (Figure

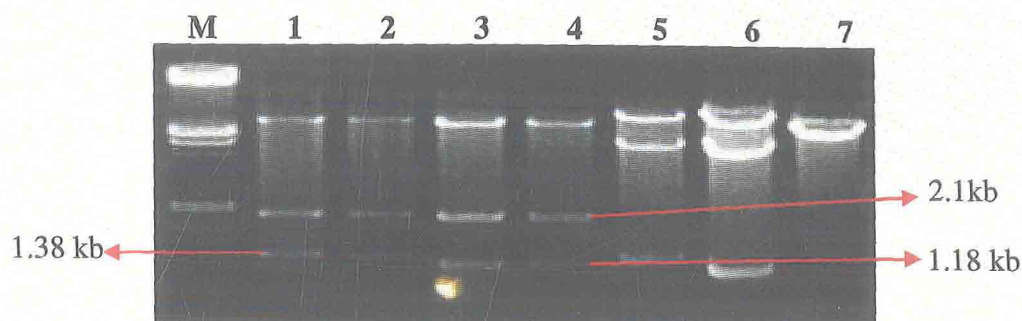


Fig. 4.53 Restriction of p-Bin-TCNN-Bar and p-Bin-TCAN-Bar clones before mobilization into *Agrobacterium* strain LBA 4404

Lanes 1 & 2. p-Bin-TCNN-Bar clones; 3 & 4. p-Bin-TCAN-Bar clones; 5. pCAMBIA1305.2-TCNN-Bar; 6. pCAMBIA1305.2-TCAN-Bar; 7. pCAMBIA0390; M. λ (HindIII+EcoRI) marker.



Fig. 4.54 Confirmation of LBA 4404:p-Bin-TCNN-Bar and LBA 4404:p-Bin-TCAN-Bar clones by restriction with *HindIII* and *EcoRI*

Lanes 1 to 4. LBA 4404:p-Bin-TCNN-Bar clones; 7 to 10. LBA4404:p-Bin-TCAN-Bar clones; 5. p-Bin-TCNN-Bar; 11. p-Bin-TCAN-Bar; 6. uncut LBA4404:p-Bin-TCNN-Bar; 12. uncut LBA4404:p-Bin-TCAN-Bar; 13. pCAMBIA0390(cut); M. λ (HindIII+EcoRI) marker.

4.54). Also plasmids from two *Agrobacterium* clones of each construct were reverse transferred into *E. coli* and further confirmed by restrictions (Figure 4.55 and 4.56) and serial PCR (Figure 4.57) analyses which confirmed the presence of all the components of the construct. Digestion with *HindIII* and *EcoRI* enzymes gave the expected 1.38 kb band for TCNN, 1.18 kb band for TCAN and the 2.1 kb band for the *bar* gene construct in both. One confirmed reverse mated clone for each of the constructs was sequenced and confirmed for the proper sequence especially the presequence and the unedited genes (coding portion of the gene cassette).

4.4.1.2 Confirmation of p-Bin-SANP and p-Bin-SAAP in LBA4404

The confirmed p-Bin-SANP and p-Bin-SAAP clones were reconfirmed by restriction with *HindIII* (Figure 4.58) after which they were mobilized into *Agrobacterium* strain LBA4404. Reverse mated *E. coli* clones were confirmed by restriction (Figure 4.59) as well as PCR (Figure 4.60). Presence of 1.1 kb and a 0.96 kb for p-Bin-SANP and p-Bin-SAAP respectively confirmed the clones. One each of the confirmed clones was sequenced and analysed to confirm the presence of *nad3* and *atp9* sequences in the antisense orientation.

4.4.1.3 Confirmation of pBin-ihp *nad3* and pBin-ihp *atp9* in LBA4404

The confirmed pBin-ihp *nad3* and pBin-ihp *atp9* clones were mobilized into *Agrobacterium* to generate LBA4404: pBin-ihp *nad3* and LBA4404: pBin-ihp *atp9* clones. The clones were reverse mated into *E. coli* and the isolated plasmids were confirmed by restriction with *HindIII* (Figure 4.61 and 4.62) and PCR with different primer combinations (Figure 4.63).

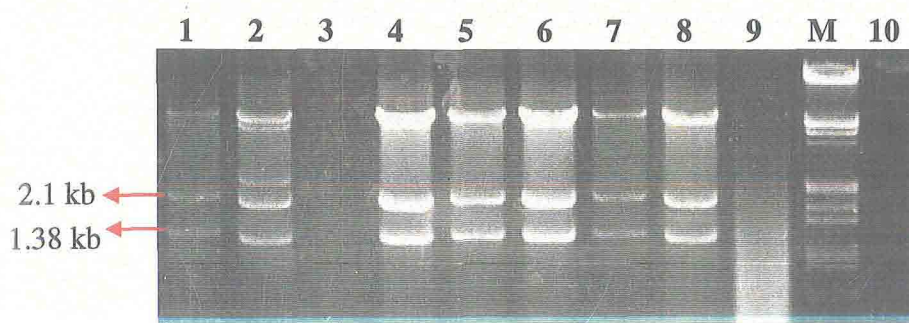


Fig. 4.55 Confirmation of reverse-mated p-Bin-TCNN-Bar clones by restriction with *Hind*III and *Eco*RI

Lanes 1 to 8. p-Bin-TCNN-Bar clones; 9. p-Bin-TCNN-Bar clone (positive control-DNA is degraded); M. λ (*Hind*III+*Eco*RI) marker; 10. pCambia0390 (negative control).

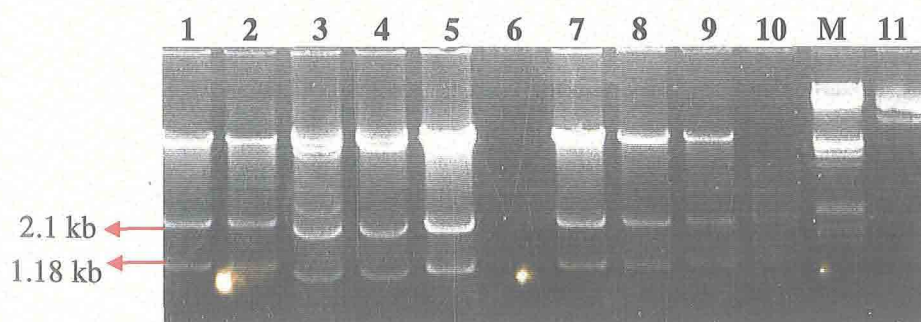


Fig. 4.56 Confirmation of reverse-mated p-Bin-TCAN-Bar clones by restriction with *Hind*III and *Eco*RI

Lanes 1 to 9. p-Bin-TCAN-Bar clones; 10. p-Bin-TCAN-Bar clone (positive control-DNA is not good); M. λ (*Hind*III+*Eco*RI) marker; 11. pCambia0390 (negative control).

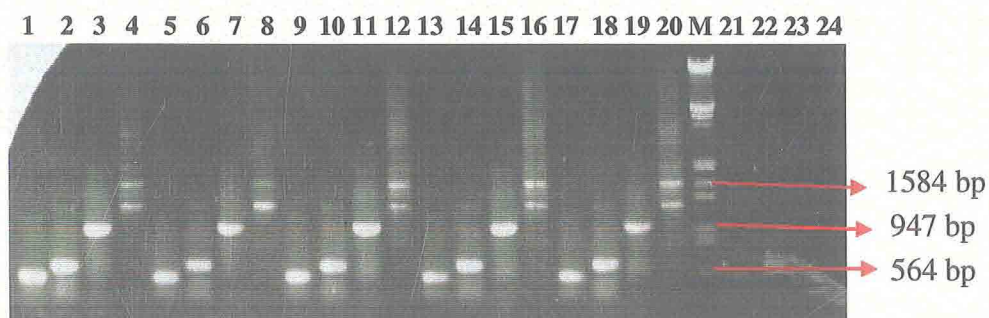


Fig. 4.57 Confirmation of LBA 4404:p-Bin-TCNN-Bar by PCR

Lanes 1,5,9,13,17. TAF+TAR primer; 2,6,10,14,18. TAF+*coxR*;
3,7,11,15,19. TAF+*nadR*; 4,8,12,16,20. TAF+*nosR*; M. λ (*Hind*III+*Eco*RI)
marker; 21 to 24. pCambia0390 (negative control).

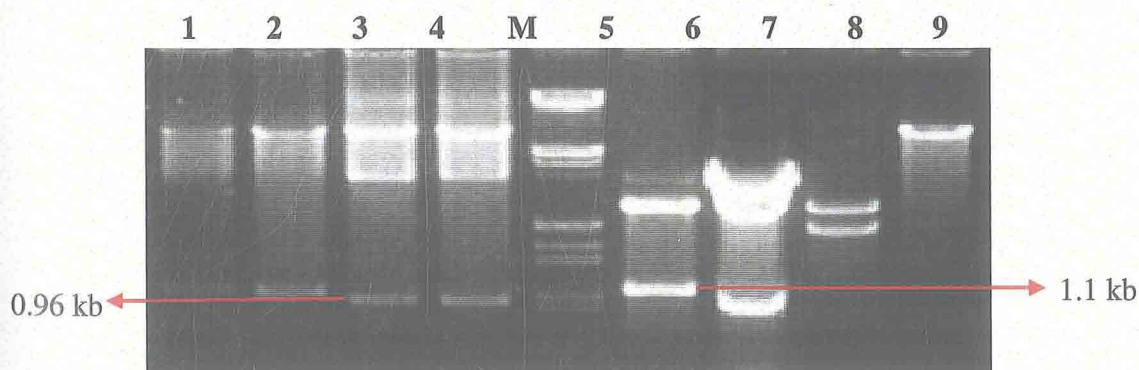


Fig. 4.58 Confirmation of pBin-SANP and pBin-SAAP clones by restriction with *Hind*III before mobilization into *Agrobacterium* strain LBA 4404

Lanes 1 & 2. pBin-SANP clones; 3 & 4. pBin-SAAP clones;
M. λ (*Hind*III+*Eco*RI) marker; 5. p-SANP; 6. p-SAAP; 7. pUC18-
Bar; 8. pCambia1300.



Fig. 4.59 Confirmation of reverse mated pBin-SANP and pBin-SAAP clones by restriction with *Hind*III

Lanes 1 to 4. reverse mated pBin-SANP clones; 7 to 11. reverse mated pBin-SAAP clones; 5. pBin-SANP; 6. p-SANP; 12. pBin-SAAP (has not got digested); 13. p-SAAP M. λ (*Hind*III+*Eco*RI) marker.



Fig. 4.60 Confirmation of reverse mated pBin-SANP and pBin-SAAP clones by PCR

Lanes 1,4,7,10,13,16,19,23. pRT seq+*nad3*F; 2,5,8,11,14,17,20,24. pRT seq+*atp9*F; 3,6,9,12,15,18,21 pRTseq+CatIntF; 4,8,12,16,20,24. pRTseq+polyAR; 1 to 3. RM pBin-SANP clone1; 4 to 6. RM pBin-SANP clone2; 7 to 9. pBin-SANP (positive control); 10 to 12. pBin-SAAP clone1; 16 to 18. pBin-SAAP clone2; 19 to 22. pCAMBIA1300 (negative control); 23 to 26. Blanks; M1. 100bp ladder; M2. λ (*Hind*III+*Eco*RI) marker.

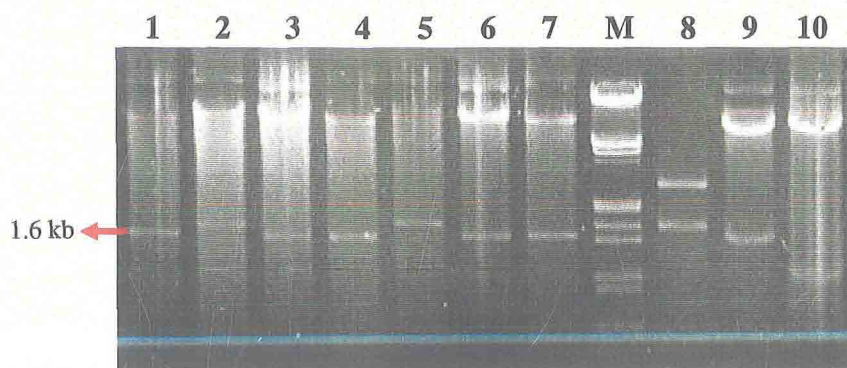


Fig. 4.61 Confirmation of reverse mated pBin-ihp *nad3* clones by restriction with *Hind*III

Lanes 1 to 7. reverse mated pBin-ihp *nad3* clones; 8. p-ihp *nad3*; 9. pBin-ihp *nad3*; 10. pRT100; M. λ (*Hind*III+*Eco*RI) marker.

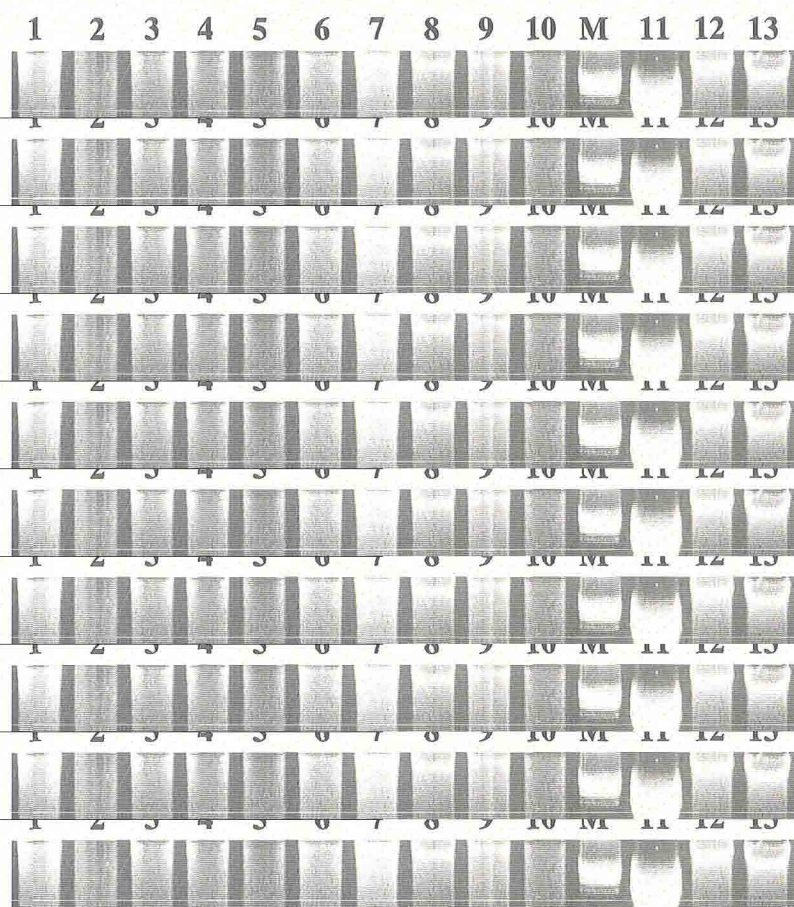




Fig. 4.63 Confirmation of reverse mated pBin-ihp *nad3* and pBin-ihp *atp9* clones by PCR

Lanes 1, 5, 9, 13, 17, 21 pRT seq + *nad3*F; 2, 6, 10, 14, 18, 22; pRT seq + *atp9*F; 3, 7, 11, 15, 19, 23 pRT100 seq + Cat Int F; 4, 8, 12, 16, 20, 24 pRT100 seq + polyAR; 1 to 4 RM pBin-ihp *nad3* clone1; 5 to 8 RM pBin-ihp *nad3* clone2; 9 to 12 pBin-ihp *nad3* (positive control); 13 to 16 RM pBin-ihp *atp9* clone1; 17 to 20 RM pBin-ihp *atp9* clone2; 21 to 24 pBin-ihp *atp9* (positive control); M₁ 100bp ladder M₂ λ (*Hind*III+*Eco*RI) marker.



Fig. 4.64 Confirmation of LBA4404: pBin-T-ihp *nad3* and LBA4404: pBin-T-ihp *atp9* clones by PCR

Upper panel TAF+Cat Int R primers; 1 to 10. LBA4404: pBin-T-ihp *atp9* clones; 12 to 17 LBA4404: pBin-T-ihp *nad3* clones; 11. pBin-T-ihp *atp9*; 18. pBin-T-ihp *nad3*; M. λ (*Hind*III+*Eco*RI) marker; 19. pCambia1300; 20. Blank

Lower Panel TAF+*nad3*R and TAF+*atp9*R; 1 to 6. LBA4404: pBin-T-ihp *nad3* clones; 7. pBin-T-ihp *nad3*; 8. pCambia1300; 9. B 10 to 19. LBA4404: pBin-T-ihp *atp9* clones; 20. pBin-T-ihp *atp9*; 21. pCambia1300.

4.4.1.4 Confirmation of pBin-T-ihp *nad3* and pBin-T-ihp *atp9* in LBA4404

The confirmed pBin-T-ihp *nad3* and pBin-T-ihp *atp9* clones were mobilized into *Agrobacterium* to generate LBA4404: pBin-T-ihp *nad3* and LBA4404: pBin-T-ihp *atp9* clones. The clones were confirmed by PCR analysis (Figure 4.64). The positive clones were reverse mated into *E. coli* and the isolated plasmids were confirmed by PCR (Figure 4.65; 4.66 and 4.67) and the orientation of the constructs in the clones was confirmed by restriction with *Xba*I enzyme (Figure 4.68).

4.4.2 Transformation of Safflower

Four of the above developed binary vectors, two each for induction of male sterility and for restoration of fertility were used for transforming safflower. Though the importance of restoration of fertility cannot be downplayed, in the present investigation induction of male sterility was the primary goal. Also, inspite of a good transformation and regeneration protocol developed in the laboratory for safflower, problems such as low frequency of sufficiently elongated shoots, reduced survival of shoots upon acclimatization and transfer to green house, sensitivity to higher temperature *etc.*, persisted. Hence it was decided to use only few constructs for safflower transformation in the present investigation. Since RNAi constructs are more efficient in gene silencing than the conventional antisense based constructs, the ihp constructs were utilized for developing the restorer lines. Since the unedited genes would be expressed under tapetal specific promoter in the male sterile lines, the restorer constructs also under TA29 promoter were used in order to have efficient silencing of the transgene. Hence the two constructs for induction of male sterility i.e.,



Fig. 4.65 Confirmation of reverse mated pBin-T-ihp *nad3* clones by PCR

Upper panel: Lanes 1 to 3. pBin-T-ihp *nad3* clone1;
 4 to 6. pBin-T-ihp *nad3* clone 2; 7 to 9. pBin-T-ihp *nad3*
 clone3; 10 to 12. pBin-T-ihp *nad3* clone4; 13 to 15. LBA4404:
 pBin-T-ihp *nad3*; M. λ (*Hind*III+*Eco*RI) marker
Lower Panel: Lanes 1 to 3. p-T-ihp *nad3*; 7 to 9. p-TA29; 4 to
 6. pCAMBIA1300; UP 1,4,7,10,13,LP 1,4,7 TAF+TAR; UP
 2,5,8,11,14; LP 2,5,8 TAF+Nad3F; UP 3,6,9,12,15 LP 3,6,9
 TAF+CatInt R.

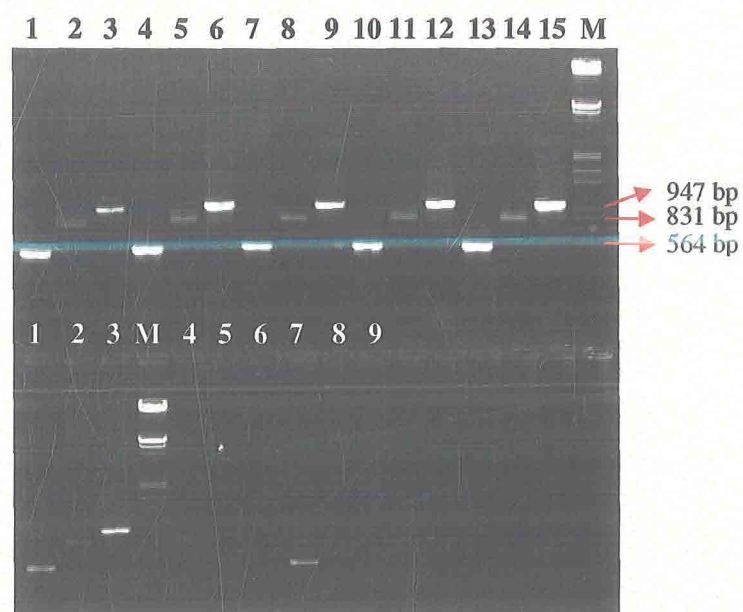


Fig. 4.66 Confirmation of reverse mated pBin-T-ihp *atp9* clones by PCR

Upper panel: Lanes 1 to 3. pBin-T-ihp *atp9* clone1; 4 to 6. pBin-T-ihp *atp9* clone 2; 7 to 9. pBin-T-ihp *atp9* clone3; 10 to 12. pBin-T-ihp *atp9* clone4; 13 to 15. LBA4404: pBin-T-ihp *nad3*; M. λ (*Hind*III+*Eco*RI) marker.

Lower Panel: Lanes 1 to 3. p-T-ihp *atp9*; 7 to 9. p-TA29; 4 to 6. pCAMBIA1300; UP 1,4,7,10,13, LP 1,4,7 TAF+TAR; UP 2,5,8,11,14 LP 2,5,8 TAF+*atp9*F UP 3,6,9,12,15 LP 3,6,9 TAF+CatInt R.



Fig. 4.67 Confirmation of reverse mated pBin-T-ihp *nad3* and pBin-T-ihp *atp9* clones by PCR

Upper panel: Cat Int F+*nad3* R: Lanes 1 to 4. pBin-T-ihp *nad3* clones; 5. LBA4404:pBin-T-ihp *nad3*; 6. p-T-ihp *nad3*; 8. pRT100; 7. pCAMBIA1300; 9. Blank; M. 100bp DNA ladder
Lower Panel: Cat Int F+*atp9* R: Lanes 1 to 4. pBin-T-ihp *atp9* clones; 5. LBA4404: pBin-T-ihp *atp9* clones; 6. p-T-ihp *atp9*; 8. pRT100; 7. pCAMBIA1300; 9. Blank.

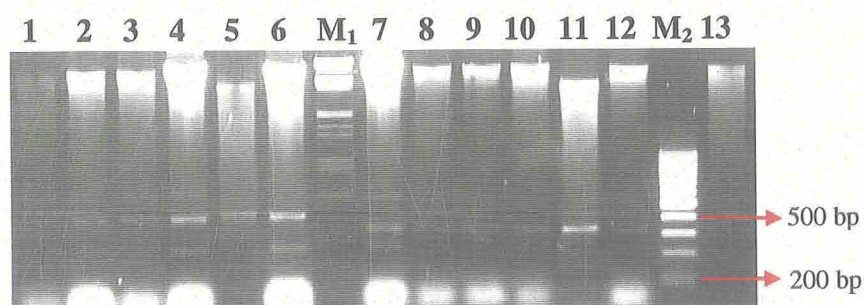


Fig. 4.68 Confirmation of orientation of the reverse mated pBin-T-ihp *nad3* and pBin-T-ihp *atp9* clones by restriction with *Xba*I

Lanes 1 to 4. RM pBin-T-ihp *nad3* clones; 7 to 10. RM pBin-T-ihp *atp9* clones; 5. p-T-ihp *nad3*; 6. pBin-TA-ihp *nad3*; 11. p-T-ihp *atp9*; 12. pBin-T-ihp *atp9*; 13. pCAMBIA1300; M₁. λ (*Hind*III+*Eco*RI) marker; M₂. 100bp ladder.

LBA4404: p-Bin-TCNN-Bar and LBA4404: p-Bin-TCAN-Bar and two of the constructs for restoration of fertility, LBA4404: pBin-T-ihp *nad3* and LBA4404: pBin-T-ihp *atp9* were used to transform safflower.

4.4.2.1 Transformation Frequency

Initially, explants of the safflower cultivar A-1 were also used along with HUS305, for transformation with the two constructs for induction of male sterility. But the transformation frequencies with A-1 hypocotyls as well as root explants were very low when compared to that with HUS305 (Table 4.8). Shoot initials were seen during culturing of explants in the selection II or selection III media (Plates 4.8, 4.9, 4.10 and 4.11). Transformation frequency was determined by calculating the ratio of the number of explants giving rise to shoots at the end of selection III to the total number of explants used for cocultivation. These results along with results with other experiments in the biotechnology laboratory (DOR), clearly showed that HUS305 was appropriate and better than other safflower cultivars for regeneration as well as transformation. Hence further experiments were conducted with only HUS305 for all the four constructs. The number of explants used per genotype, total number of explants used, number of explants responding after each selection and the final transformation frequency for each of the constructs are given in Table 4.8. The number of hypocotyl explants used ranged from 242 to 1022 and root explants ranged from 172 to 536 for HUS305 transformation. Hypocotyl explants responded better than root explants as the transformation frequencies ranged from 8.7% to 34.5% for HUS305 hypocotyl explants whereas for root explants the transformation frequency ranged from 2.9% to 19.8%. Among the four constructs used, the highest transformation frequency for both the

Table 4.8 Transformation frequency in safflower for the different constructs

Construct	Genotype	No. of co-cultivations	Explant used	Total no. of explants	No. of explants responding after SI	No. of explants responding after SII	No. of explants responding after SIII	Transformation frequency (%)
LBA4404: p-Bin-TCNN-Bar	HUS-305	14	Hypocotyls	1022	492 (48.1)	306 (29.9)	170	16.6
			Roots	536	311 (58.0)	55 (10.3)	29	5.4
A-1	HUS-305	3	Hypocotyls	297	110 (37.0)	63 (21.2)	37	12.5
			Roots	412	106 (25.7)	29 (7.0)	26	6.3
LBA4404: p-Bin-TCAN-Bar	HUS-305	10	Hypocotyls	498	354 (71.1)	219 (44.0)	172	34.5
			Roots	172	136 (79.1)	120 (11.6)	34	19.8
A-1	HUS-305	3	Hypocotyls	242	100 (41.3)	33 (13.6)	3	1.2
			Roots	418	232 (55.5)	1 (0.2)	0	0
LBA4404: pBin-T-ihp <i>nad3</i>	HUS-305	8	Hypocotyls	548	289 (52.7)	180 (32.9)	165	30.1
			Roots	256	79 (30.9)	36 (14.1)	47	18.4
LBA4404: pBin-T-ihp <i>atp9</i>	HUS-305	4	Hypocotyls	429	178 (41.5)	164 (38.2)	38	8.9
			Roots	234	44 (18.8)	51 (21.8)	7	3.0

* Figures in paranthesis are percentages

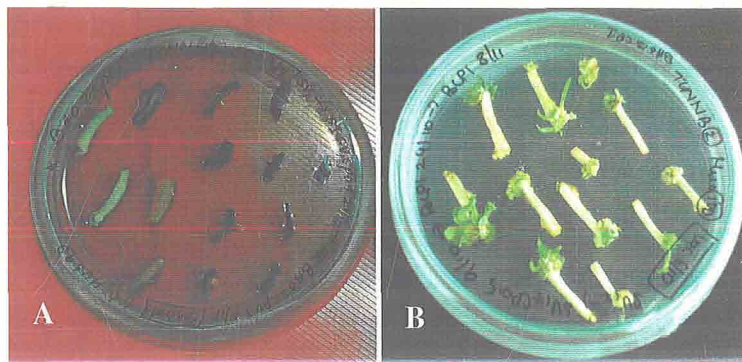


Plate 4.8 Shoots induced from safflower explants transformed with TCNN-Bar construct

- A - Untransformed control explants on SII medium
- B - Transformed hypocotyls explants giving rise to shoots in SII medium



Plate 4.9 Enlarged view of shoots arising from transformed hypocotyl explants

- A - Multiple shoots arising from a hypocotyl explant
- B - Single shoot arising out of a hypocotyl explant

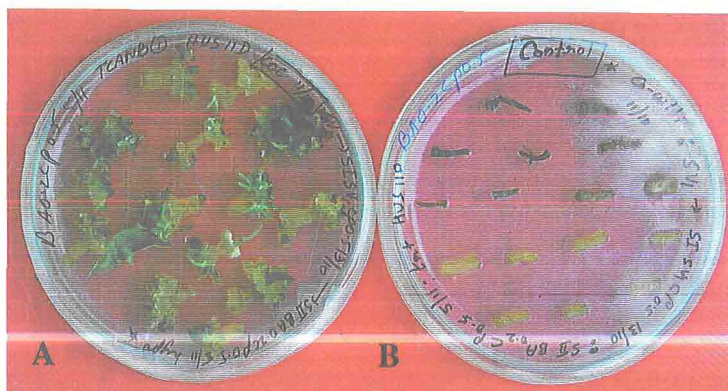


Plate 4.10 Shoots induced from safflower explants transformed with TCAN-Bar construct

- A - Transformed hypocotyls explants giving rise to shoots on SII medium
- B - Untransformed control explants on SII medium

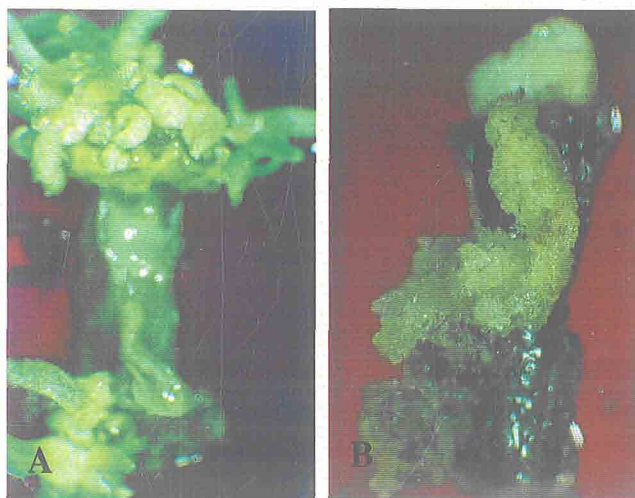


Plate 4.11 Response of transformed safflower root explants (TCAN-Bar) on selection medium

- A - Shoots arising from a transformed root explant
- B - Control untransformed root explants

explants was seen with LBA4404: p-Bin-TCAN-Bar whereas the lowest was seen with LBA4404: pBin-T-ihp *atp9*.

4.4.2.2 Elongation of the transformed shoots

The total number of safflower shoots obtained ranged from 35 (LBA4404: pBin-TA-ihp *atp9*) to 161 (LBA4404: p-Bin-TCNN-Bar). The shoots that had formed by the end of the third selection were maintained on shoot multiplication medium (MS powder + 1x CaCl₂ + BA 0.5 mg/l or BA 0.2 mg/l) for 1 to 3 cycles of 15 days each. Some shoots elongated in this medium, which were directly transferred into rooting media. Some other shoots showed elongation upon transfer to medium containing kinetin (MS powder + 1x CaCl₂ + kinetin 0.5 mg/l) before they were transferred to rooting medium. But a general observation during this study was that very few shoots showed higher elongation (i.e 3-4 nodes or with longer internodal distance) (Plate 4.12). Most of the shoots showed less number of nodes and shorter internodal length. However for the purpose of rooting, we considered those shoots that put forth at least two nodes (even upon several subcultures on the maintenance/elongation medium) as elongated. Different media used are listed in Table 3.12.

4.4.2.3 Rooting of the transformed shoots

Two rooting media combinations were used in the present study initially (RM₁-Half MS + IBA 2.0 mg/l + Phloroglucinol 2.0 mg/l and RM₂-Half MS + NAA 0.5 mg/l). Though shoots on both the media gave rise to roots, the frequency of root induction was higher when RM₂ was used. Hence subsequently only RM₂ was used for rooting (Table 4.9). Among the four constructs, shoots with LBA4404: p-Bin-TCNN-Bar showed the highest rooting frequency (60.3%). Roots induced on RM₁ were thin and long whereas

Table 4.9 Frequency of root induction in the safflower transgenics

Construct	No. of shoots transferred to RM ₁	No. of shoots responded on RM ₁	Frequency of root induction on RM ₁ (%)	No. of shoots transferred to RM ₂	No. of shoots responded on RM ₂	Frequency of root induction on RM ₂ (%)
LBA4404: p-Bin-TCNN-Bar	32	12	37.5	68	41	60.3
LBA4404: p-Bin-TCAN-Bar	36	10	27.8	70	30	42.8
LBA4404: pBin-T-ihp <i>nad3</i>	-	-	-	31	12	38.7
LBA4404: pBin-T-ihp <i>atp9</i>	-	-	-	6	3	50

Table 4.10 Details of the safflower transgenics

Construct	Total no. of shoots	No. of shoots elongated	No. of shoots rooted	No. of shoots acclimatized	No. of shoots that reached flowering	Pollen fertility of the obtained shoots
LBA4404: pBin-TCNN-Bar	161	108	53	53	6	3 PS 3 F
LBA4404: pBin-TCAN-Bar	110	106	40	36	5	1PS 4 F
LBA4404: pBin-T-ihp <i>nad3</i>	101	33	12	12	-	-
LBA4404: pBin-T-ihp <i>atp9</i>	35	8	3	3	-	-

PS – Partial sterile F – Completely fertile

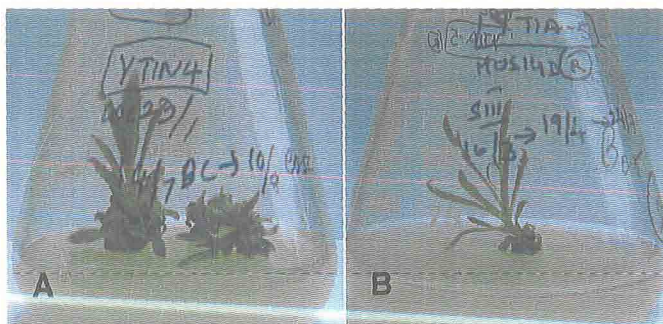


Plate 4.12 Elongation of transgenic T-ihp-*nad3* (A) and T-ihp-*atp9* (B) safflower shoots

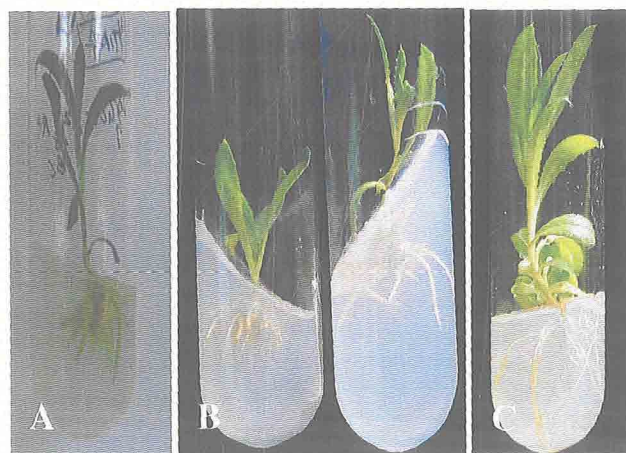


Plate 4.13 Root induction in Safflower transgenic shoots

A & B-Response in RM1 medium
C - Response in RM2 medium

those induced on RM₂ were thicker and shorter in length (Plate 4.13).

4.4.2.4 Hardening and Acclimatization

Most of the rooted shoots (barring some that were lost due to contamination) were acclimatized (as mentioned in section 3.3.2.6 of chapter III) (Plates 4.14, 4.15, 4.16 and 4.17) But very few survived upto the stage of flowering. The shoots were not sturdy and appeared very weak and lanky. Only six shoots obtained with LBA4404: p-Bin-TCNN-Bar and five shoots with LBA4404: p-Bin-TCAN-Bar reached flowering stage. The few shoots obtained with LBA4404: pBin-T-ihp *nad3* and LBA4404: pBin-T-ihp *atp9* did not survive at all (Table 4.10).

4.4.3 Transformation of tobacco

To assess the efficacy of the constructs developed we opted to use the model system tobacco. Two constructs for induction of male sterility *i.e.*, LBA4404: p-Bin-TCNN-Bar and LBA4404: p-Bin-TCAN-Bar and two of the constructs for restoration of fertility, LBA4404: pBin-T-ihp *nad3* and LBA4404: pBin-T-ihp *atp9* were used to transform tobacco by the standard leaf disc transformation procedure. Cocultivation with each of the *Agrobacterium* clones was done as mentioned in Chapter III. The transformed explants were subjected to three selections of 15 days in appropriate media supplemented with either hygromycin or phosphinothricin (Plates 4.18, 4.19 and 4.20). The transformation frequency for tobacco ranged from 12.94% (LBA4404: p-Bin-TCNN-Bar) to 47.83% (LBA4404: pBin-T-ihp *nad3*) (Table 4.11). As in the case of safflower, the shoots arising at the end of third selection were

Table 4.11 Transformation frequency in tobacco for the different constructs

Construct	No. of co-cultivations	Total no. of explants	No. of explants responding after SI	No. of explants responding after SII	No. of explants responding after SIII	Transformation frequency
LBA4404:p-Bin-TCNN-Bar	2	171	115 (67.3%)	86 (50.3%)	62	36.3%
LBA4404:p-Bin-TCAN-Bar	3	170	162 (95.3%)	45 (26.5%)	22	12.9%
LBA4404: pBin-T-ihp <i>nad3</i>	2	23	16 (69.6%)	12 (52.2%)	11	47.8%
LBA4404: pBin-T-ihp <i>atp9</i>	2	33	29 (87.9%)	19 (65.5%)	14	42.4%

Table 4.12 Details of the tobacco transgenics

Construct	Total no. of shoots	No. of shoots rooted	No. of shoots acclimatized	No. of shoots that reached flowering	Pollen fertility of the obtained shoots
LBA4404: pBin-TCNN-Bar	58	30	30	29	PS/F
LBA4404: pBin-TCAN-Bar	17	17	10	7	AF
LBA4404: pBin-T-ihp <i>nad3</i>	11	11	11	11	AF
LBA4404: pBin-T-ihp <i>atp9</i>	12	12	12	12	AF

PS – Partial sterile F – Completely fertile AF – All plants completely fertile



Plate 4.14 Hardening of safflower plants: Plants covered with plastic sheets upto 5-7 days after acclimatization (Tissue Culture lab) vermiculite pots



Plate 4.15 Hardening of safflower plants: Plants covered with plastic sheets cut at the corners upto 10-14 days after acclimatization (Tissue Culture lab)



Plate 4.16 Hardening of safflower plants: Plastic sheets removed after 10-14 days after acclimatization and plants kept as such for 5-7 days (Tissue Culture lab)



Plate 4.17 Hardening of safflower plants: Plants initially in smaller vermiculite pots transferred to green house.

A and B -transgenic plants
C - Control untransformed plant



Plate 4.18 Shoot induction in tobacco explants transformed with TCNN-Bar construct

- A - Untransformed tobacco explants (negative control)
- B - Transformed explants giving rise to shoots in phosphinothricin containing medium



Plate 4.19 Response of transformed tobacco explants transformed with T-ihp *nad3* construct

- A - Control explants in hygromycin containing medium
- B - Transgenic T-ihp *nad3* shoots regenerating from hygromycin containing medium

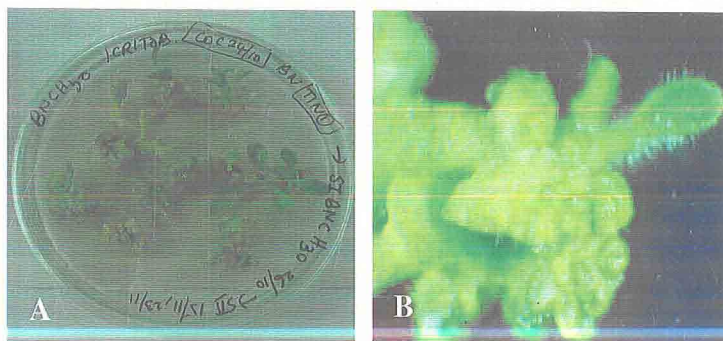


Plate 4.20 Shoot induction in tobacco explants transformed with T-ihp *nad3* construct

- A - Transformed explants giving rise to shoots in hygromycin containing medium
- B - Enlarged view of a developing transgenic shoot

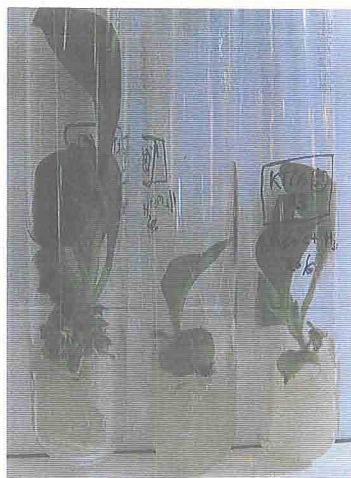


Plate 4.21 Root induction and elongation of tobacco transgenic shoots

maintained on the shoot multiplication medium (MS powder + 1x CaCl₂ + BA0.5 mg/l). Individual shoots were then transferred on to half strength MS medium which served as both rooting and elongation medium, as the shoots elongated and also put forth roots simultaneously (Plate 4.21). All the tobacco shoots transferred to this medium rooted and most of them were acclimatized initially in tissue culture lab (Plates 4.22) and then in green house (Plate 4.23 and 4.24) (except those that were lost due to contamination). All the acclimatized tobacco shoots survived and grew up to flowering stage (unlike safflower shoots) (Table 4.12). The plants were normal in morphology and were comparable to the untransformed tobacco plants in all the phenotypic characters.

4.5 Confirmation of the developed safflower and tobacco transgenics

All the developed safflower and tobacco transgenics were analysed for the presence of the transgene by PCR analysis as well as herbicide/antibiotic sensitivity assay. For the shoots with TCNN-Bar and TCAN-Bar that reached flowering stage, pollen fertility was also analyzed.

4.5.1 PCR Analysis for the presence of the transgenes

All the safflower and tobacco plants that were acclimatized as well as several plants at the tissue culture level (which were sufficiently grown in maintenance and elongation medium) were analyzed for the presence of the transgene construct by PCR (Tables 4.13, 4.14, 4.15, 4.16). Most of the plants at this stage were PCR positive while the plants that were PCR negative invariably showed sensitivity to the herbicide/antibiotic (Plates 4.25, 4.26 and 4.27). Some plants which were classified as positive during PCR analysis also showed sensitivity to the selection agent indicating perhaps the differential expression of the introduced gene.

Table 4.13 PCR analysis of transgenic shoots at the tissue culture level

Plant	Safflower		Tobacco	
Construct	Number of shoots*	PCR positive shoots	Total number of shoots*	PCR positive shoots
LBA4404:p-Bin-TCNN-Bar	80	25	70	41
LBA4404:p-Bin-TCAN-Bar	65	13	17	4
LBA4404: pBin-T-ihp <i>nad3</i>	19	14	10	9
LBA4404: pBin-T-ihp <i>atp9</i>	9	6	12	12

*Only shoots from those transformants from which sufficient leaves could be collected carefully were used in this study

Table 4.14 Analysis of flowered safflower TCNN-Bar and TCAN-Bar transgenics

Construct	Transgenic Plant No.	PCR for the transgene (+/-)	Reaction for Phosphinothricin Sensitivity	Pollen fertility (%)
LBA4404: p-Bin-TCNN-Bar	35	+	ND	34.2
	87-3	-	S	99.0
	76-6	+	R	40.0
	76-4	+	R	99.0
	87-2	+	R	98.0
	NN 2	+	R	69.0
LBA4404: p-Bin-TCAN-Bar	21-1	-	ND	99.0
	B-2	+	R	99.0
	69-3	+	ND	99.0
	69-2	-	S	98.0
	70-1	+	R	32.0

R-Resistant S- Susceptible ND-Not determined

Table 4.15 Analysis of flowered tobacco TCNN-Bar transgenics

Sl. No.	Transgenic Plant No.	PCR for transgene (+/-)	Reaction for PPT Sensitivity	Pollen fertility (%)
1	1-1	+	R	98-99
2	11-3	+	R	58.4
3	17-2	+	R	98-99
4	17-4(1)	+	R	98-99
5	17-4(2)	+	R	98-99
6	17-6	+	CS	98-99
7	20	+	R	98-99
8	22-1	+	CS	98-99
9	23-2	+	R	98-99
10	24-2	+	R	98-99
11	51	+	MS	98-99
12	58(1)	+	R	98-99
13	58(2)	+	R	98-99
14	73-2	+	R	98-99
15	78-1	+	R	98-99
16	78-2	+	R	49.6
17	78-4	+	R	24.7
18	79(1)	+	R	98-99
19	79(2)	+	R	98-99
20	87-1	+	R	98-99
21	96-2	+	R	98-99
22	96-3	+	R	22.0
23	97-1	+	R	98-99
24	99-2	+	R	65.6
25	99-4	+	R	68.4
26	99-5	+	R	98-99
27	106-1	+	R	76.8
28	107-2	+	R	98-99
29	99-1	+	R	5.0
30	A2	+	R	98-99

R-Resistant CS-Completely Susceptible MS-Moderately Susceptible

Table 4.16 Analysis of flowered tobacco TCAN-Bar transgenics

Construct	Transgenic Plant No.	PCR for the transgene (+/-)	Reaction for Phosphinothricin Sensitivity	Pollen fertility (%)
LBA4404: p-Bin-TCAN-Bar	2	-	CS	98-99
	3(1)	+	R	98-99
	3(2)	+	R	98-99
	4	-	CS	98-99
	7	-	CS	98-99
	8(1)	+	MS	98-99
	8(2)	+	R	98-99

R-Resistant CS-Completely Susceptible MS-Moderately Susceptible

Table 4.17 Analysis of flowered tobacco T-ihp-*nad3* transgenics

Construct	Transgenic Plant No.	PCR for the transgene (+/-)	Reaction for Hygromycin Sensitivity	Pollen fertility (%)
LBA4404: p-Bin-T-ihp- <i>nad3</i>	3	+	R	98-99
	20	-	CS	98-99
	24	+	R	98-99
	27	+	MS	98-99
	28	+	R	98-99
	29	+	CS	98-99
	57	+	CS	98-99

R-Resistant
CS-Completely
Susceptible

MS-Moderately Susceptible

Table 4.18 Analysis of flowered tobacco T-ihp-*atp9* transgenics

Construct	Transgenic Plant No.	PCR for the transgene (+/-)	Reaction for Hygromycin Sensitivity	Pollen fertility (%)
LBA4404: p-Bin-T-ihp- <i>atp9</i>	1	+	R	98-99
	4-1	+	ND	98-99
	8	-	R	98-99
	12	+	CS	98-99
	15	-	MS	98-99
	24	-	R	98-99
	25	-	R	98-99
	29	-	R	98-99
	39	-	R	98-99
	40	+	ND	98-99
	41	-	R	98-99
	46	+	R	98-99

R-Resistant CS-Completely Susceptible MS-Moderately Susceptible



Plate 4.22 Hardening of tobacco transgenic plants:

A - Plants covered with plastic sheets upto 10-14 days after acclimatization (Tissue Culture lab)

B - Plant after removing plastic cover (Tissue Culture lab)



Plate 4.23 Hardening of tobacco transgenic plants:
Plant in small vermiculite pots hardened in green house for 7-10 days

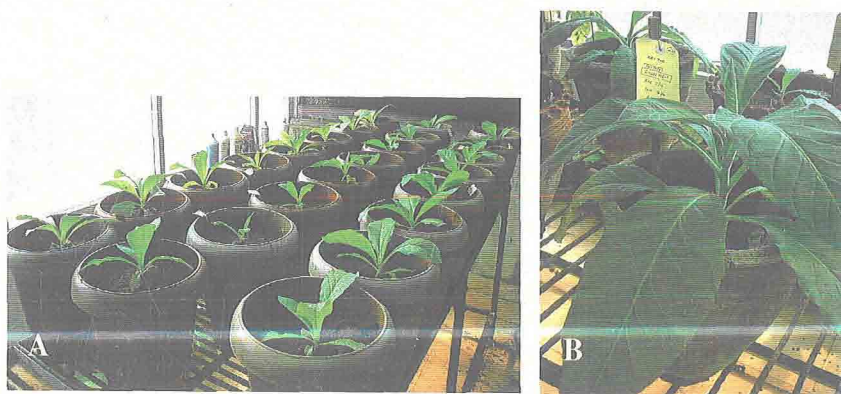
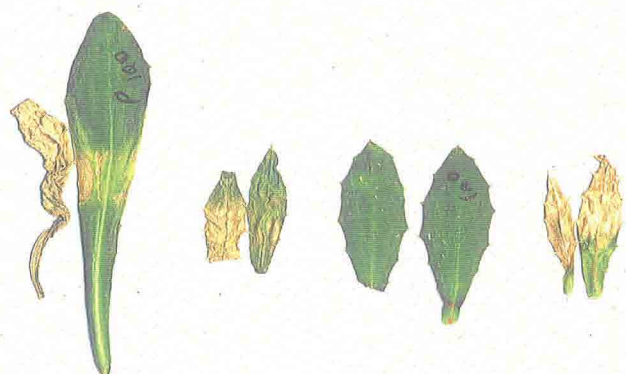


Plate 4.24 Hardening of tobacco transgenic plants:

Plant transferred into larger soil filled pots

A - Tobacco transgenic plants in green house

B - A tobacco plant growing in the green house



**Control
Seedling**

**Tissue
Culture
control**

**PCR +ve
TCAN Bar
Transgenic**

**PCR -ve
TCAN Bar
Transgenic**

Plate 4.25 Safflower leaf assay for Phosphinothricin sensitivity

In each case, 5 μ l of PPT was placed on the leaf

Left: 1 mg/ml concentration Right: 0.1 mg/ml concentration

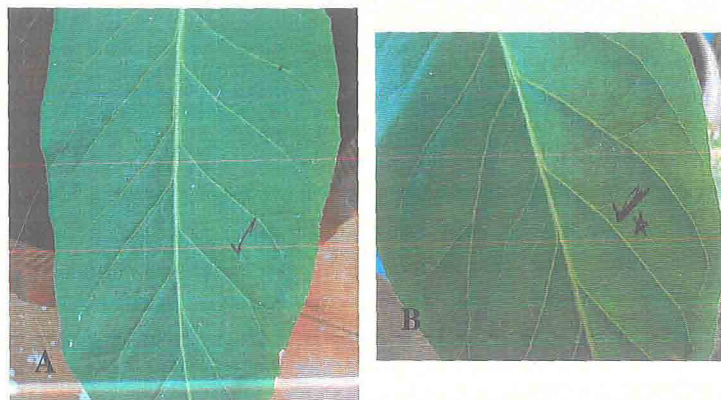


Plate 4.26 Tobacco transgenic plants showing resistance to Phosphinothricin and Hygromycin

A - TCNN-BAR plant showing resistance to PPT (5 μ l of 1mg/ml conc.)

B - T-ihp-atp9 plant showing resistance to Hyg (5 μ l of 15mg/ml conc.)

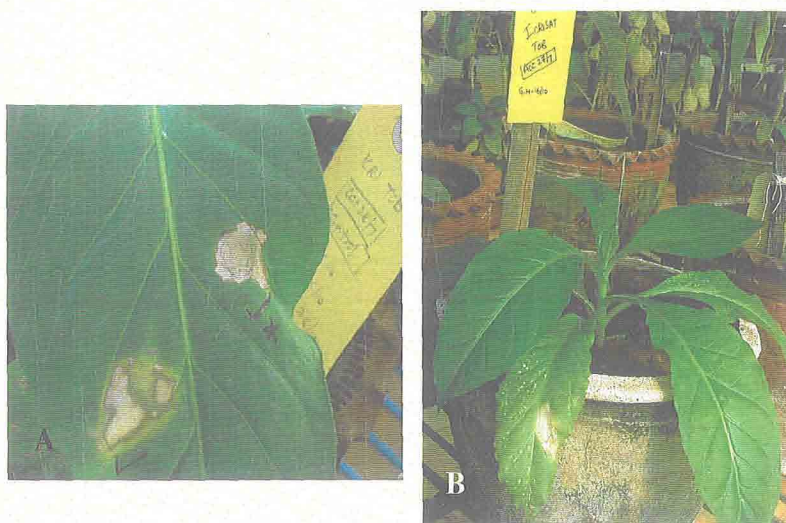


Plate 4.27 Control tobacco plants showing susceptible reaction to Phosphinothricin and Hygromycin

A - Control untransformed plant susceptible to PPT as well as Hyg

B - A control tobacco plant showing PPT susceptible reaction

The presence of TCNN and TCAN transgenes in the plants were confirmed by using appropriate sets of primers. Presence of intact TA29 promoter and *coxIV* presequence downstream of this promoter was verified using TA29 F and *coxIV* R primers which gave a 630 bp band with both set of plants (TCNN-Bar and TCAN-Bar). Further presence of *nad3* and *atp9* downstream of *coxIV* presequence was confirmed by using TA29 F and the gene specific reverse primers. Both the PCRs gave expected size amplicons (990bp with TCNN-Bar plants and 900 bp band with TCAN-Bar plants) (Figures 4.69, 4.70, 4.71 and 4.72). Use of just the gene specific primers for PCR was not taken up as that would amplify the endogenous/native *nad3* and *atp9* genes.

The presence of the T-ihp *nad3* construct in the transgenic tobacco and safflower plants was confirmed by a 1.1kb amplicon obtained with TAF and CIR primers and 565 bp band with CIF and *nad3R* primers (Figures 4.73 and 4.75). T-ihp *atp9* constructs in the transgenic plants were confirmed by 1.0kb band with TAF and CIR primers and 465bp band with CIF and *nad3R* primers (Figures 4.74 and 4.76). All the PCR reactions were repeated twice to confirm the transgenic nature of the plants.

At the green house level, majority of the plants were PCR positive for the transgene. This was because a preliminary PCR analysis was done with the putative transgenic safflower and tobacco shoots at the tissue culture level. Sufficient care was taken to avoid contamination while collection of leaf material and only those shoots that had sufficiently grown were used for this analysis. The results of this analysis indicated that only some of the shoots actually carried the transgene (safflower shoots TCNN-Bar: 25/80, TCAN-Bar: 13/65; tobacco shoots TCNN-Bar: 41/70, TCAN-Bar: 4/17) indicating that a large number of escapes were present in both safflower and tobacco (Table 4.13).



Fig. 4.69 Confirmation of transgenic safflower TCNN-Bar plants by PCR

Upper Panel TAF+coxIVR: Lanes 1,3. positive transgenic plants (~630 bp band); 2, 4 to 11. negative transgenic plants

Lower Panel TAF+nad3R: 1,3. positive transgenic plants (~990 bp band); 2, 4 to 11. negative transgenic plants; **Both panels:** 12. pBin-TCNN-Bar (positive control); 13. pCAMBIA0390 (negative control); 14. Blank.

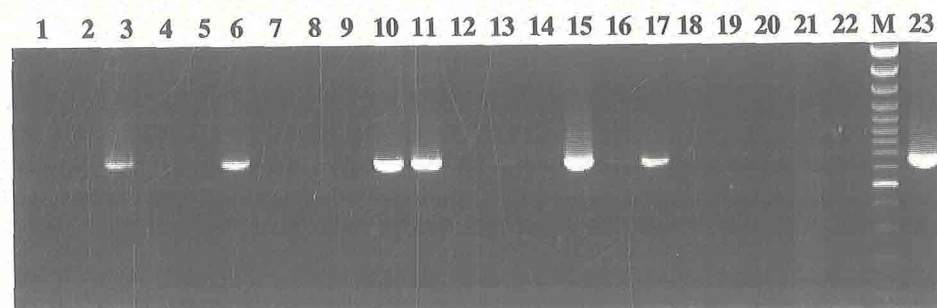


Fig. 4.70 Confirmation of transgenic safflower TCAN-Bar plants by PCR
Primers: TAF and CoxIVR primers (645 bp)

Lanes 1 to 22. Transgenic plants; 23. pBin-TCNN-Bar (positive control); M. 100bp DNA ladder.



Fig. 4.71 Confirmation of transgenic tobacco TCNN-Bar plants by PCR

Primers: TAF+*nad3R*: Lanes 1 to 4, 6, 7, 9 to 18. positive transgenic plants (~990 bp band); 5,8. negative transgenic plants; 19. pBin-TCNN-Bar (positive control).

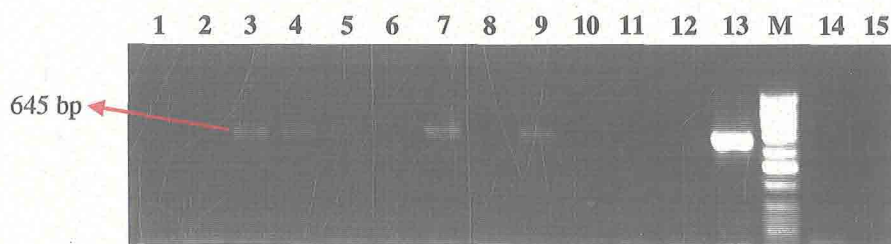


Fig. 4.72 Confirmation of transgenic tobacco TCAN-Bar plants by PCR

Primers: TAF+*coxIVR* (630 bp): Lanes 1 to 4, 6, 7, 9 to 18. positive transgenic plants (~990 bp); 5,8. negative transgenic plants; 19. pBin-TCAN-Bar (positive control).

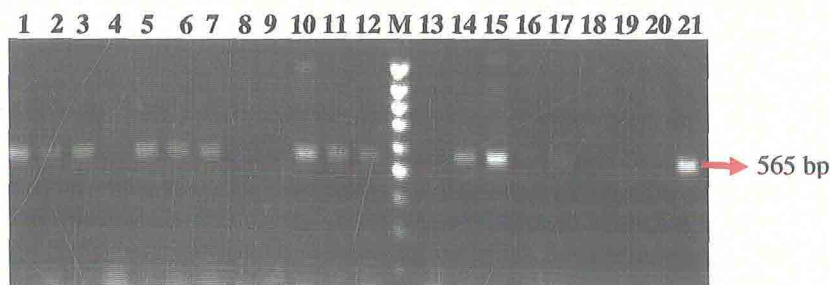


Fig. 4.73 Confirmation of transgenic safflower T-ihp-*nad3* plants by PCR

Primers: CatIntF + *nad3*R: Lanes 1,2,3,5,6,7,10,11,12,14,15,17,19. positive transgenic plants (~565 bp); 4,8,9,13,16,18,21. negative transgenic plants; 21. pBin-T-ihp-*nad3* (positive control).



Fig. 4.74 Confirmation of transgenic safflower T-ihp-*atp9* plants by PCR

Primers: CatIntF + *atp9*R: Lanes 1,3,4,5,6,8. positive transgenic plants (~470 bp); 2, 7, 9. negative transgenic plants; 10. pBin-T-ihp-*atp9* (positive control); 11. pCambia1300 (negative control); 12. Blank.

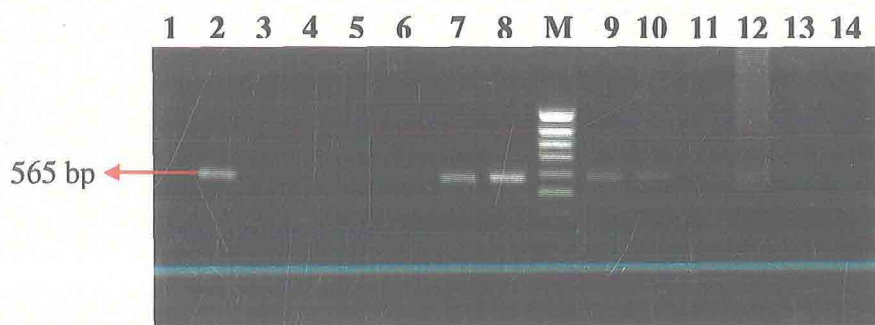


Fig. 4.75 Confirmation of tobacco T-ihp-*nad3* transgenics by PCR

Primers: CatInt F+*nad3*R: Lanes 2,7,8,9,10. positive transgenic plants; 3,4,5,6,11. negative transgenic plants; 12. pBin-T-ihp-*nad3* (positive control); 13. pCambia1300 (negative control); 14. Blank.

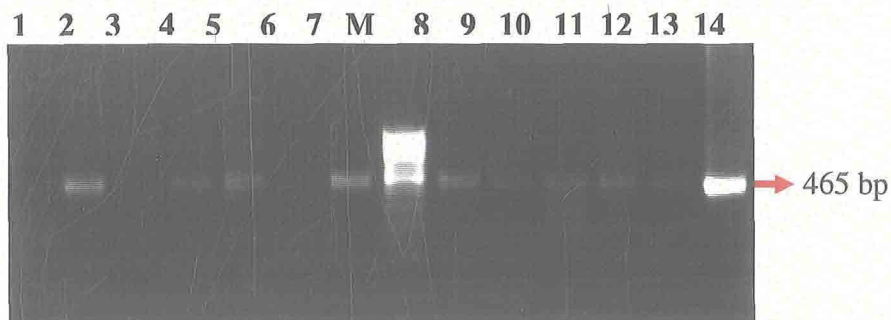


Fig. 4.76 Confirmation of transgenic tobacco T-ihp-*atp9* plants by PCR

Primers: CatIntF + *atp9*R: Lanes 2 to 12. positive transgenic plants; 13. pBin-T-ihp-*atp9* (positive control).

4.5.2 Assay for Phosphinothricin/Hygromycin sensitivity

Since the transgene constructs for induction of male sterility *i.e* LBA4404: p-Bin-TCNN-Bar and LBA4404: p-Bin-TCAN-Bar also included the bar gene cassette that confers resistance to the herbicide phosphinothricin, the plants harbouring these transgenes are expected to show resistance to phosphinothricin. Hence assay was carried out on the leaves of all the acclimatized transgenic safflower and tobacco plants to check for the phosphinothricin resistance in these plants. Similarly, since the constructs developed for restoration of fertility (LBA4404: pBin-T-ihp *nad3* and LBA4404: pBin-T-ihp *atp9*) have the hygromycin resistance gene cassette within the T-DNA, the plants with these constructs were tested for hygromycin sensitivity. Based on the response, the plants were classified as resistant, moderately susceptible or completely susceptible to phosphinothricin/hygromycin. Tables 4.14, 4.15 and 4.16 show the results of the Phosphinothricin/Hygromycin assay done for the safflower as well as tobacco plants in the green house that reached the flowering stage.

4.5.3 Classification of transgenic plants with respect to male sterility

The safflower transgenic plants were classified based on the pollen reaction to 0.1% acetocarmine stain. The plants which showed more than 20% sterile pollen (which failed to take up the acetocarmine stain) were classified as 'partial sterile' whereas the remaining were considered 'fertile' plants.

In case of tobacco, the classification was based on pollen staining as well as the seed set. Those plants which showed more than 20% sterile pollen but formed capsules were classified as 'partial sterile' and the plant (99-1) that showed 95 % sterile pollen and did not

form capsules was designated as 'Male sterile' plant. The remaining plants that formed normal capsules and had more than 90% fertile pollen were termed 'fertile' plants.

4.5.4 General phenotype of the plants

All the transgenic safflower and tobacco plants were observed for any differences with respect to vegetative traits like plant height, number of leaves, number of flowers *etc.* Two types of control safflower plants were raised; one type included untransformed plants, which originated from tissue culture explants and the other type included those from seeds sown directly in pots in the green house. All the tissue culture raised safflower shoots (transgenics and control) were lanky and twiny compared to the seed sown control plants indicating the inherent problems in *de novo* shoot regeneration (Plate 4.28). In case of tobacco plants there was no difference in the morphological traits between the controls and the transformed plants with all the constructs.

4.5.5 Study of the floral morphology

All the safflower and tobacco plants that reached the flowering stage (Tables 4.14, 4.15, 4.16) were observed closely for any abnormalities with respect to the floral morphology.

Very few of the safflower plants had survived upto the flowering stage. All the tissue culture raised plants had a very small flower bud with less florets compared to the control plants raised from seeds (Plate 4.29). In case of tobacco, the transgenic TCNN-Bar plants that showed sterility (Plate 4.32) had small sized flower buds and showed severe flower drop. Three of these plants had flowers with protruded stigma having regressed anthers



Plate 4.28 Flowering of hardened safflower transgenic shoots



Plate 4.29 Flowers of transgenic (A) vs Control untransformed (B) safflower

(below the level of stigma) and very less visible pollen whereas in the control as well as other transgenic plants, anthers were always either above stigma or at the same level as stigma (with abundant pollen) (Plate 4.31). The single male sterile TCNN-Bar tobacco plant (99-1) did not show any abnormality and also did not show any visible pollen. All the flowers of this plant dropped except those which were pollinated with pollen from control plants and such crossed flowers set seeds indicating that the plant was female fertile. All the tobacco transgenics developed for fertility restoration had normal flowers resembling the untransformed control plants.

4.5.6 Pollen studies

Both pollen staining as well pollen germination studies were carried out under the present investigation.

4.5.6.1 Pollen staining

Anthers were crushed to release the pollen, stained with 1% acetocarmine stain and viewed under light microscope after 3-4 hours. The fertile pollen were thick, uniform shaped and pink coloured having picked up the stain whereas the sterile ones were yellow with irregular or malformed shape (Plate 4.30). Among the six TCNN-Bar safflower transgenic plants that reached flowering, three showed partial sterility (34.2% to 69% pollen fertility) whereas one out of the four flowered TCAN-Bar safflower plants showed partial sterility (32% pollen fertility) (Table 4.14).

Among the 30 TCNN-Bar tobacco plants, 7 showed partial sterility (24.7 to 76.8% pollen fertility) (Plate 4.32) (Table 4.15). One TCNN-Bar plant (99-1), showed less than 5%

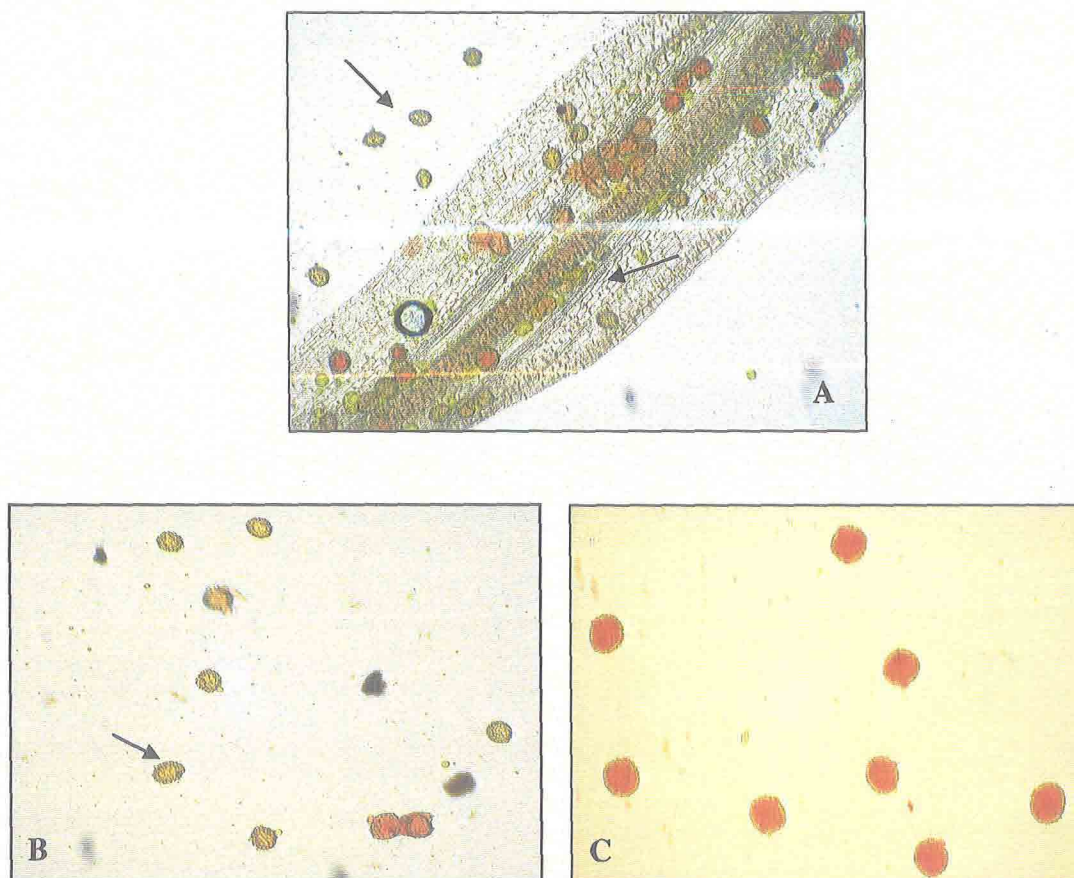


Plate 4.30 Partial fertility in a safflower TCNN-Bar transgenic plant (No. 35)

A and B - Pollen grains from partially fertile transgenic plant

C - Pollen grains from a control untransformed plant

Arrows indicate sterile pollen grains

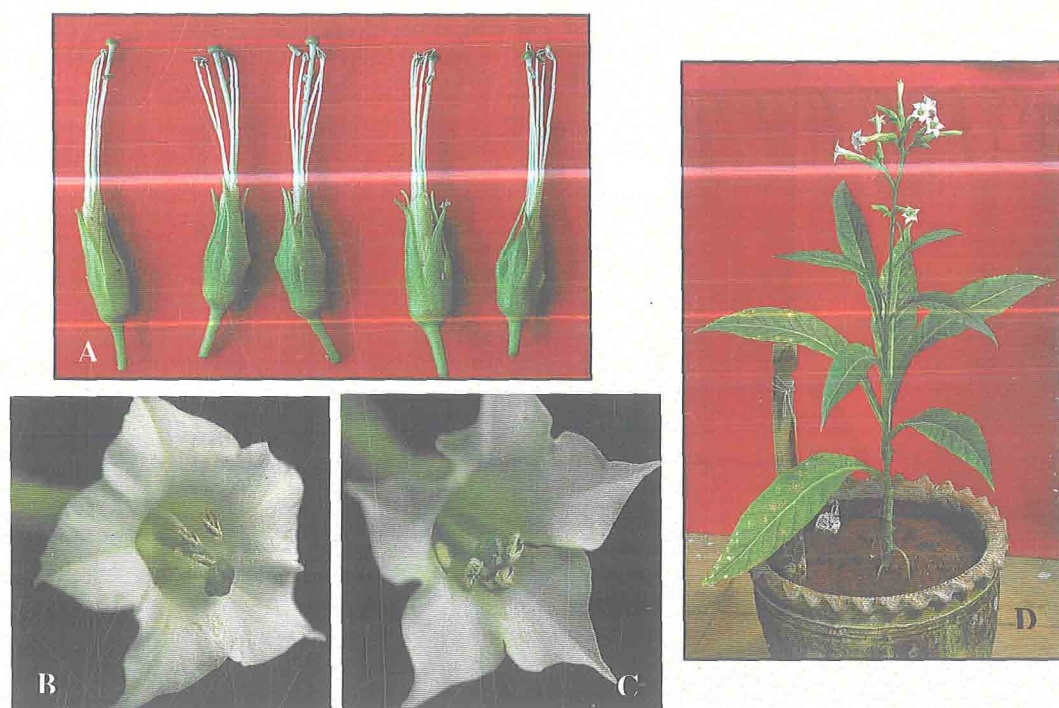


Plate 4.31 Floral morphology of the partially fertile TCNN-Bar tobacco transgenic

- A - Transgenic (first three) and control flowers (petals removed)
- B - A single flower of a partial sterile transgenic plant
- C - A single flower of a control untransformed plant
- D - A transgenic plant reached flowering stage

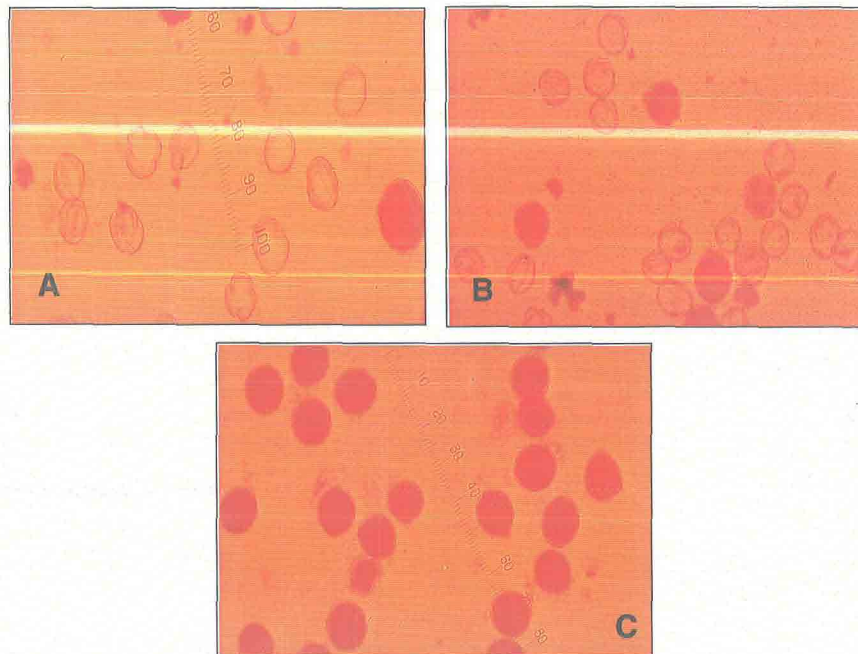


Plate 4.32 Partial fertility in tobacco TCNN-Bar transgenic plants stained with Acetocarmine (1%).

A and B show sterile and fertile pollen on two of the transgenic tobacco plants whereas C indicates fertile pollen in a control untransformed plant

darkly stained pollen. The rest of the plants with the constructs for male sterility induction and all the transgenic plants for restoration of fertility showed more than 90% fertility and their pollen on staining with acetocarmine resembled that of the control untransformed plants (Table 4.14 to 4.16).

4.5.6.2 Pollen germination studies

Since it was expected that the partial sterile safflower plants would set seeds, it was decided not to disturb the florets and hence the pollen germination studies were not carried out for safflower. But pollen germination studies were carried out for tobacco TCNN-Bar plants. The pollen of the partial sterile plants, showed severe abnormalities during growth in comparison to the control pollen which showed normal growth of pollen tubes (Plate 4.33) and none of the few pollen seen in the male sterile plant (99-1) germinated.

4.5.7 Seed/Capsule formation

All the tobacco transgenic plants, except the TCNN-Bar male sterile plant 99-1, formed capsules and set seeds (selfed) though the capsule size (Plate 4.34) and the quantity of seeds in the partial sterile plants were less compared to control and the completely fertile plants. The male sterile plant (99-1) that showed severe flower drop, formed normal capsules and set seeds when pollinated by pollen from control plants indicating normal female fertility (Plate 4.35). The tobacco plants with the restoration constructs formed capsules and seeds (selfed) normally like control plants. In case of safflower, except one TCAN-Bar transgenic plant (which had showed complete fertility) that set two seeds (selfed), no other safflower plant set seeds despite visibly having pollen. But many of the

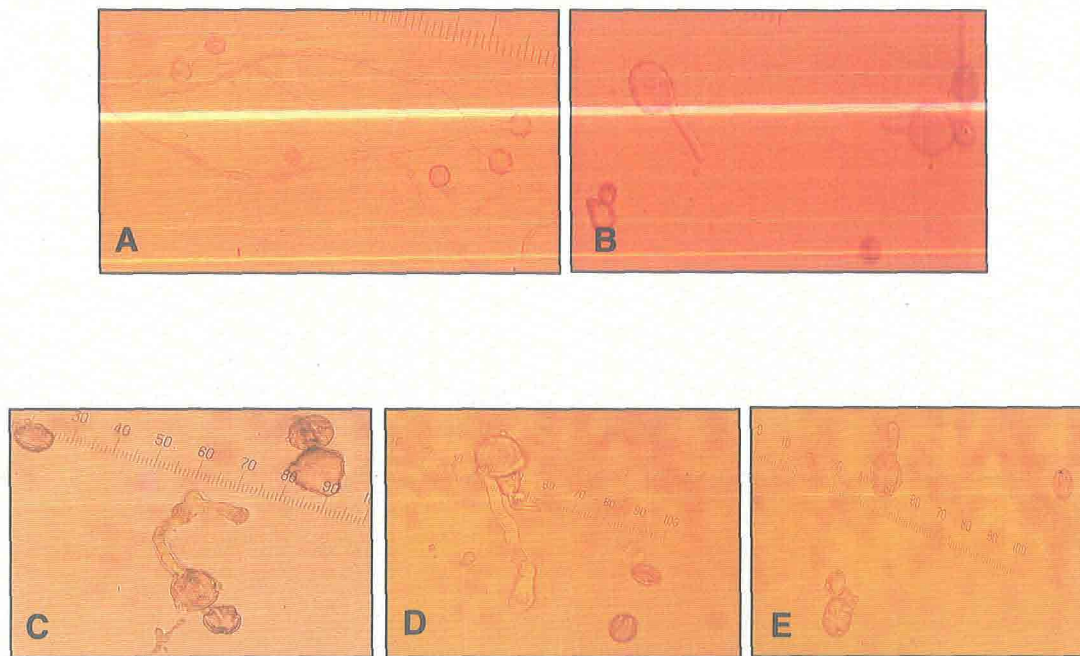


Plate 4.33 Pollen germination in Partial sterile tobacco plants:

A- Pollen germination of control untransformed tobacco plants

B,C,D,E - Abnormal pollen germination in transgenic tobacco
TCNN-Bar plants

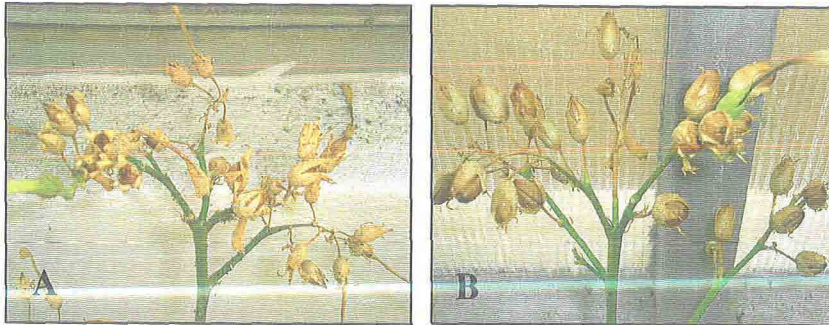


Plate 4.34 Capsules of TCNN-Bar transgenic tobacco plants

A- Partially sterile - did set seeds but capsules were smaller in size compared to control plant (B)

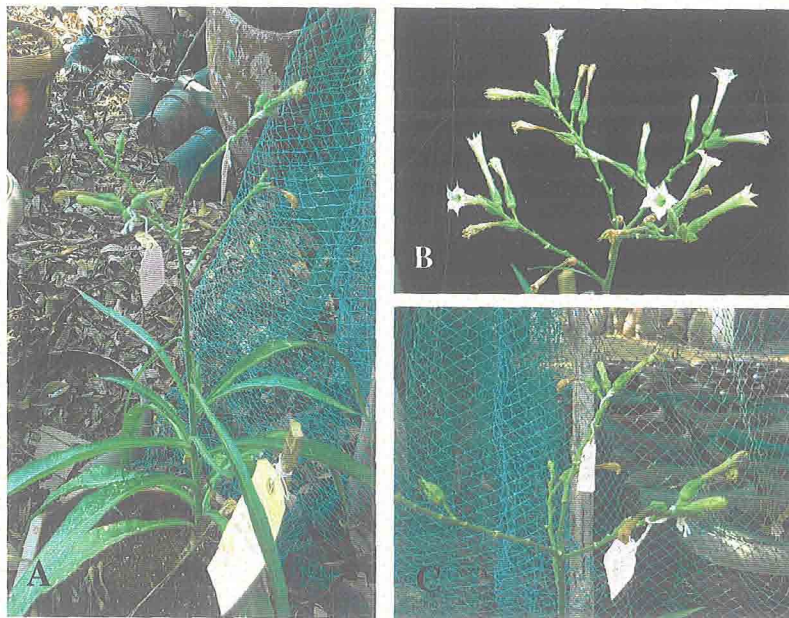


Plate 4.35 TCNN-Bar transgenic tobacco plant (99-1) showing severe flower drop

A - TCNN-Bar transgenic tobacco plant; Several flowers were formed on this plant initially (B) - but there was severe flower drop(C)

tissue culture raised control plants also failed to set seeds indicating that this could be due to the inherent problems associated with regenerated plants that were very weak.

In summary, the present study included identification of candidate genes through RNA editing studies, standardization of safflower regeneration through immature embryos, development of gene constructs (for male sterility induction as well as fertility restoration), development of transgenic safflower and tobacco plants with these constructs and their confirmation. This is the first report of RNA editing in the mitochondrial genes of safflower. Though *u-atp9* gene (from wheat) has been used in a similar attempt for induction of male sterility, the *u-nad3* gene has been used for the first time in this study for male sterility induction. Though the number of transgenic plants generated are small to confirm the action of these transgenes, the partial sterile plants obtained in both safflower and tobacco and the male sterile plant obtained in tobacco are preliminary indicators of the proof-of-concept that *u-nad3* as a candidate gene could lead to male sterility.

The indications obtained in this study is that, for induction of male sterility with these constructs, as a future line of work, more number of transgenic plants will have to be raised with both the constructs (*u-atp9* and *u-nad3*) to validate the utility of these constructs and to establish the proof-of-concept that these candidate genes could induce male sterility. The progenies of both partial sterile plants and complete male sterile plant obtained in this study will have to be analyzed at genetic, molecular and morphological level. Molecular analysis could include confirmatory analyses like southern blotting to determine the transgene copy number, northern and/or western blotting to confirm the expression of the transgene *etc.* need to be done to further confirm the transgene integration and its expression. Also the T₁ seeds of the transgenics for fertility restoration, generated under this study, need to be sown and T₂

plants have to be analysed to study the segregation pattern of the transgene, in order to study its stable integration. For the successful development of a transgenic system to be exploited commercially, a large number of independent transformation events generating stable transgenic plants with optimum levels of expression of the gene of interest are needed to exercise the selection of plants with normal phenotype for all the other agronomic and morphological traits. For this purpose further modification in the safflower transformation and regeneration protocols are needed so as to overcome the problems faced during the present investigation and to achieve the ultimate goal of realizing a good transgenic male sterility-restoration system.

CHAPTER V

DISCUSSION

CHAPTER V

DISCUSSION

A good hybrid seed production system is needed to exploit the high levels of heterosis reported in safflower. Though GMS and CGMS systems have been tried in safflower, they are hindered by several inherent problems. At this juncture, transgenic approach offers a viable alternative to develop male sterile and restorer lines in this crop. Transgenic male sterility by exploiting the process of RNA editing has already been demonstrated. When unedited version of *atp9* gene from wheat was expressed in the nucleus and the resultant protein was targeted into mitochondria, it led to ablation of tapetum leading to male sterility in tobacco and *Arabidopsis* (Hernould *et al.*, 1993; Gomez-Casati *et al.*, 2002). Based on this logic, in the present investigation, studies were undertaken to identify important candidate mitochondrial genes that undergo extensive RNA editing. The unedited versions of these genes were then used to develop constructs for induction of male sterility as well as cognate constructs for fertility restoration. These constructs were used to transform safflower and tobacco explants using *Agrobacterium* mediated genetic transformation method. Transgenic plants were developed and analyzed. The background for the present investigation, the rationale for the formulation of the project, the results obtained, their relevance and the future line of work are discussed below.

5.1 Male sterility systems in safflower

Safflower is an important oilseed crop, grown widely in recent years for its edible oil that is rich in poly-unsaturated fatty acids. Safflower has been reported to have very high level of heterosis (227% for seed yield and 80% for oil production), which can be harnessed,

to increase the yield and oil content, by development of a good hybrid seed production system. Hybrids, apart from producing better yields, are more uniform and more adaptable to the vagaries of nature. To effect out crossing (for hybrid seed production), male sterility in the plants used as female parent is essential to realize the economic benefits of a good hybrid seed production system. Though GMS and CGMS system are being used in some crops for commercial hybrid seed production, there are inherent problems associated with these systems. A single CGMS system for safflower reported in USA and being exploited there, is still not available in our country. Though till date four GMS based safflower hybrids have been released in India, farmers are still dependent on varieties as the problems with the hybrid seed production based on GMS system, make them economically unviable. Hence, transgenic approach offers a viable alternative in developing a good male sterility-restoration system in safflower.

5.2 Transgenic male sterility

Induction of transgenic male sterility has been achieved by following different approaches. In all these attempts, disruption of the male gametophyte development or the release of pollen grains have been the targets. The different approaches mainly adopted are expression of cytotoxic genes, antisense inhibition of important genes, inducing conditional male sterility, male sterility by inducing hormonal imbalances, male sterility by alteration of metabolic processes, expressing male sterility causing genes from heterologous systems, exploitation of cellular processes like RNA editing and more recently male sterility by chloroplast engineering. Despite several reports of transgenic male sterility by these various approaches (Table 2.2), only the barnase-barstar system has been successful at a commercial

level and has been tested in many systems like tobacco, oilseed rape, cauliflower, tomato, cotton, corn, wheat, chicory *etc.* (Banga *et al.*, 2006). Another approach involving exploitation of RNA editing phenomenon for induction of male sterility has been demonstrated in different plant systems like tobacco, *Arabidopsis* and rice (Hernould *et al.* 1993; Gomez-Casati *et al.*, 2002; http://www.avesthagen.com/docs/newsletters/jan_feb_2005.pdf). Among the various methods described above, those which use DNA sequences derived from plant sources such as use of CMS inducing gene from heterologous system as well as exploiting innate cellular processes such as RNA editing pose little concern with respect to biosafety compared to the expression of cytotoxic genes derived from different organisms.

Several factors like selection of suitable gene(s), the selection of appropriate promoters, the suitable vectors to be used (cloning vectors as well as binary vectors), the selection marker and other sequences (like presequences) need to be considered before development of gene constructs for induction of transgenic male sterility.

The careful selection of gene(s) used for development of appropriate constructs is the most important step. Though the gene(s) chosen depends on the approach being used to induce male sterility, it is better that it should preferably not have any pleiotrophic effect, should not be cytotoxic of serious nature and preferably must be of plant origin. Another important factor is the use of the most appropriate promoter to regulate the expression of the introduced gene(s) spatially and temporally. Since the intention is to target the cells important for male gametogenesis, anther/tapetal tissue specific/pollen specific promoters are most suitable. But use of pollen specific promoter leads to gametophytic male sterility

which is difficult to adapt for exploitation of hybrid vigour whereas anther/tapetal specific promoters would lead to sporophytic male sterility, which is more desirable.

Also many of the approaches to induce male sterility, aim at disruption of the mitochondrial functioning. This is because disruption of mitochondrial functioning would reduce/affect the energy (ATP) production and even slight reduction in ATP synthesis severely affects the male gametogenesis or pollen fertility. As procedures for transforming chondriomes are not available, if any proteins are to be used to disrupt mitochondrial functioning, they must be produced in the cytoplasm and targeted into mitochondria. To achieve this, mitochondrial target sequences are fused to the 5' portion of the protein to be targeted into mitochondria. *coxIV* presequence from yeast has already been successfully used to target the in-frame *U-ATP9* into mitochondria in tobacco and *Arabidopsis* (Hernould *et al.*, 1993; Gomez-Casati *et al.*, 2002).

In the present investigation, it was intended to induce male sterility by expressing the unedited version of mitochondrial genes from safflower in the tapetal cell layer using TA29 promoter from tobacco and targeting the resultant proteins into mitochondria using the *coxIV* transit peptide sequence from yeast. The tissue specificity of this promoter has been well demonstrated in many plants belonging to different genera (Mariani *et al.*, 1990, 1992; Kriete *et al.*, 1996; Cho *et al.*, 2001). At the Directorate of Oilseeds Research, the tapetal tissue specific expression pattern of TA29 promoter and the ability of the *coxIV* presequence to target the in-frame fusion proteins into mitochondria had been established in safflower by using the *gus* reporter system (Kalyani *et al.*, 2007).

Initially, experiments were carried out to identify the suitable mitochondrial gene(s) that undergo substantial editing so that these gene(s) could be used for developing constructs in safflower.

5.3 Identification of mitochondrial genes that undergo substantial RNA editing in safflower

RNA editing leads to modification of RNA through nucleotide insertion, deletion or modification. RNA editing in plant mitochondria usually results in a protein with a sequence that is more conserved across different plants. The importance of editing in the formation of functional proteins has been hypothesized by several investigators (Gualberto *et al.* 1989; Araya *et al.* 1992). The hypothesis was further confirmed when it was shown that mitochondrial targeting of the polypeptide, translated from unedited version of *atp9* and *nad9* transcripts, leads to male sterility (Hernould *et al.* 1993; http://www.avesthagen.com/docs/newsletters/jan_feb_2005.pdf). The suppression of the introduced *u-atp9* gene by adopting antisense strategy resulted in the restoration of male fertility (Zabaleta *et al.*, 1996) further establishing that unedited version of mitochondrial genes disrupt normal functioning of the cell.

5.3.1 RNA editing in *atp6*

Initially *atp6* was selected as the candidate gene because in crops like sorghum, rice and *petunia* editing of *atp6* transcripts has been reported to be important for fertility. The interaction of RNA processing and editing of B-*atp6* transcripts has also been found to play a role in controlling CMS expression and the restoration of fertility in rice (Iwabuchi *et al.*,

1993). In sorghum, a transcript-specific loss of RNA editing was observed in anthers of CMS lines (Howad and Kempken 1997; Howad *et al.*, 1998). Loss of *atp6* RNA editing, as it occurs in sorghum CMS anthers, has been found to mimic mutations in human mitochondrial diseases. Mutations at specific positions in the *atp6* gene are the cause of neurogenic muscle weakness, ataxia and retinitis pigmentosa (NARP or Leigh syndrome) (Kempken *et al.*, 1998). Though these reports encouraged utilizing *atp6* for sterility induction, results on editing of *atp6* gene transcripts in safflower and sunflower, in the present investigation, were not encouraging. The full-length *atp6* genomic and cDNA clones of safflower could not be obtained as only ~250 bp band was consistently obtained during PCR and RT-PCR even after repeating the reactions, whereas a 906 bp band in both was expected. 5' RACE (Rapid Amplification of cDNA ends) that is usually employed to determine the 5' end of the transcript region was attempted using two sets of nested primers. Cloning of the resultant product in T/A vector and sequencing the clones revealed that the sequence obtained was not that of *atp6*. Hence this study could not elucidate the full-length *atp6* cDNA sequence in safflower. But it was possible to develop full-length *atp6* cDNA clones for sunflower. Comparison of the genomic and cDNA *atp6* sequences in sunflower revealed only two changes in the transcripts. These results suggested that *atp6* may not be a suitable candidate gene for this approach and hence this gene was not used further.

5.3.2 Editing in *nad3* and *atp9*

An intensive literature search was done to identify important mitochondrial genes based on the criteria that they should be important for the functionality of mitochondria and should undergo substantial editing. This would establish their potential as candidate genes

for inducing male sterility through exploitation of RNA editing phenomenon. The two genes *nad3* and *atp9* were identified as *nad3* codes for subunit 3 of the mitochondrial NADH oxidoreductase complex and unedited *atp9* has been used successfully to induce male sterility in tobacco and *Arabidopsis* (Hernould *et al.*, 1993; Gomez-Casati *et al.*, 2002). These two genes, apart from coding for important mitochondrial proteins, have also been reported to have substantial editing in other crops. Also another advantage was that these two were intron-less genes, which facilitated easier analysis of these genes especially because the genomic (unedited) versions were of interest for developing the constructs. Therefore, it was intended to study the editing pattern of these two genes in safflower.

Results of the present investigation indicated that safflower *nad3* transcripts undergo editing at a total of 19 sites including 12 complete and 7 partial edit sites which led to 14 amino acid changes. The *nad3* gene sequence is conserved in majority of the species and RNA editing in *nad3* has been reported in several crops like sunflower (Perrotta *et al.*, 1996), *Oenothera lamarckiana* (Schuster *et al.*, 1990), Carrot (Rurek *et al.*, 2001), wheat (Gualberto *et al.*, 1991), sorghum (Howard and Kempken, 1997), *Petunia hybrida* (Lu and Hanson, 1992) *etc.* Most of these studies involved development of genomic and cDNA clones and sequence comparisons to detect the edited sites. Gualberto *et al.*, 1991 have studied the presence of several partially edited *nad3-rps12* transcripts in wheat, but found that only the mature and completely edited transcripts are used for translation indicating that the edited transcripts are the ones forming the functional protein. Rurek *et al.*, (2001) reported differences in editing of *nad3* transcripts from CMS and fertile carrots, though the differences were with respect to partial and silent edit sites. The number of edited sites in

nad3 transcript of higher plants reported till date ranges from three sites in rice (Suzuki *et al.*, 1991) to 21 sites in wheat (Gualberto *et al.*, 1991).

The *atp9* gene codes for subunit 9 of the ATPase complex which is a membrane-associated protein subunit involved in the formation of proton-translocating F_0 portion of the ATPase in mitochondria. RNA editing in the *atp9* gene of several plants has been reported. The present study has revealed that safflower *atp9* transcripts undergo editing at a total of 12 sites of which 10 are complete and two are partial edit sites leading to 8 amino acid substitutions. Editing in *atp9* transcript resulted in shortening of the ATP9 polypeptide by 12 aminoacids due to a stop codon that was created by editing at nucleotide position 223. This editing was seen in all the safflower clones sequenced, highlighting the importance of the shortening in length of ATP9. This has also been observed in case of *Oenothera* (Schuster and Brennicke, 1990), wheat (Begu *et al.*, 1990), petunia (Wintz and Hanson, 1991), sorghum (Salazar *et al.*, 1991), potato and tobacco (Hernould *et al.*, 1992). But in other crops like soybean (Pesce and Grabau, 1993), the stop codon at the position 223 is already genomically encoded. Begu *et al.*, 1990 have sequenced the wheat ATP9 protein at the carboxyterminal region and confirmed that only the edited mRNA is translated into protein that further stresses the importance of editing of *atp9* transcripts. Though the *atp9* gene sequences of some species including safflower is longer, the creation of stop codon due to editing at nucleotide position 223, results in an equal polypeptide length (74 amino acids) for ATP9 across species. Hence, editing at this site probably helps in maintaining a conserved carboxy terminal in ATP9 protein across species. It could be deduced that any additional amino acids at the 3' end may decrease or hinder the proper functioning of this protein. If so, the RNA editing might be a mechanism to correct such defects and improve

their functionality. As observed for *nad3* transcripts, all the edit sites of *atp9* transcripts that effected amino acid substitutions were also conserved in majority of the species studied so far. The comparison of the DNA, RNA and deduced protein sequences in the present study with those of other species clearly indicated that the substantial RNA editing enhanced the degree of conservation of the safflower NAD3 and ATP9 protein

Comparison of the relative editing efficiency of different edit sites indicated that the editing was more stringent whenever it led to amino acid changes in the resultant proteins and not so when the editing *per se* did not change the amino acids due to codon degeneracy or wobbling at the 3rd position of the codon. It was also noted that complete edit sites were conserved in most of the species while partial edit sites were not. It was interesting to note that the editing at particular sites was not conserved across plants if already the genomic DNA encoded the required amino acid, making editing superfluous. For example, in *atp9* transcripts, C to U editing at position 82 brings about Leucine (L) to Phenylalanine (F) change in safflower. But in rapeseed, *Arabidopsis* and soybean, where there is no editing for this site, the genomic DNA already codes for phenylalanine. The observation that partially edited clones were quite preponderant in the steady state pools of transcripts of both *nad3* and *atp9*, place these into the second category of transcripts as defined by Lu and Hanson (1996) where transcripts are heterogeneous with respect to the editing extent and the partially edited transcripts are readily detectable and sometimes are more abundant than completely edited ones. It was interesting to note that *nad3* transcripts of *Petunia*, *Oenothera* and carrot (Gualberto *et al.* 1989; Schuster *et al.* 1990; Wissinger *et al.* 1990; Lu and Hanson, 1996; Rurek *et al.* 2001) also belonged to this category. Although the experimentally detected partially edited transcripts are thought to be intermediates in the

editing process, there is as yet no experimental or empirical proof that all incompletely edited transcripts do become fully edited. Also, it remains to be investigated whether the partially edited transcripts are utilized by the translation machinery or whether only completely edited transcripts are chosen for translation. This could be determined only by direct sequencing of NAD3 and ATP9 proteins. The significance of partial editing on the expression of the genes analyzed in different plants so far is not clear, though such studies have already been carried out with human mitochondrial genes (Mass and Rich, 2000). It could be that partial editing of transcripts may help plant mitochondria achieve protein sequence diversity allowing the flexibility to cope with environmental fluctuations.

PREP-Mt, a recent software for prediction of edited sites (Mower, 2005) could predict 16/19 sites of *nad3* (11 complete and 5 partial) and 9/12 sites of *atp9* (all complete). As this software is dependent on the conserved nature of editing for a particular transcript across different species, the ability to rightly predict most of the edit sites in both *nad3* and *atp9* transcripts of safflower, again testify that editing process is evolved to produce conserved proteins. However, some edit sites were not identified by the software indicating the importance of empirical determination of editing in the transcript of interest.

Comparison of the deduced amino acid sequences of NAD3 and ATP9, before and after editing, across species clearly indicated that RNA editing led to proteins which are highly conserved in nature and the editing process reduces the percentage differences in amino acid sequences of proteins. For both NAD3 and ATP9 proteins, the differences reduced significantly after the editing thereby signifying the importance of editing in attaining highly conserved primary sequence of the proteins perhaps leading to the most

stable form of the protein with least free energy. This observation was more apparent for ATP9 proteins where the percentage differences reduced to near zero after editing.

In *Arabidopsis thaliana*, study of the complete mitochondrial genome sequence, has revealed that the general tendency of RNA editing is to increase the proportion of hydrophobic amino acid codons, the three most frequent amino acid transitions being Serine (S) to Leucine (L), Proline (P) to Leucine (L) and Serine (S) to Phenylalanine (F) (Giege and Brennicke, 1999). Though it is not clear why editing facilitates incorporation of these selected amino acids, it might be opined that these amino acids might be important for the functioning of the proteins. It is known that Leucine is used exclusively in protein and enzyme construction whereas phenylalanine is a common aromatic amino acid found in many proteins. In our studies, in both *nad3* and *atp9* transcripts of safflower, all the substituted amino acids due to RNA editing were hydrophobic and predominantly Leucine and Phenylalanine. The analysis of deduced NAD3 and ATP9 sequences, further confirmed the increased hydrophobicity of both the proteins due to editing. As most of the mitochondrial proteins are membrane bound and to be membrane bound the proteins need to be more hydrophobic, it is logical to think that editing process might have evolved so that more hydrophobic amino acids are incorporated in place of hydrophilic ones to facilitate incorporation of the protein into membrane. This hypothesis was further strengthened by the results obtained using transmembrane helix prediction software, HMMTOP. The analysis indicated two transmembrane helices for ATP9 and three helices for NAD3. It was interesting to note that the amino acid changes brought about by editing were mainly located within the transmembrane helices for both the proteins. It was interesting to note that the

reduced portion of the ATP9 protein at the carboxy-terminal contained mainly hydrophilic amino acids.

From these experiments it was concluded that extensive editing takes place in the transcripts of both *atp9* and *nad3* genes of safflower and these edit sites are conserved across the species. This high degree of conservation of the length and composition of both ATP9 and NAD3 across species, as a result of their transcript editing, indicated the functional importance of RNA editing for these proteins. To the best of our knowledge, this is the first report of RNA editing in safflower. The extensive and conserved patterns of editing observed in these two transcripts imply that editing at these sites might be crucial for the functionality of NAD3 and ATP9 proteins. A preliminary *in silico* analysis (TargetP software) of the translational fusion of unedited version of NAD3 and ATP9 with *coxIV* pre-sequence from yeast indicated that they are targeted into mitochondria and are membrane bound giving credence to the hypothesis that these two proteins could be translocated into mitochondria and get docked in the mitochondrial membrane. Hence, constructs were developed for induction of male sterility as well as restoration of fertility with these two genes.

5.4 Development of constructs for induction of male sterility

The vector developed earlier in the Biotechnology laboratory of the Directorate of Oilseeds Research, Hyderabad, was used as the basal vector to develop the gene constructs in the present investigation. This vector (pCAMBIA1305.2-TCON) had the gene cassette TCON with the component sequences TA29 promoter from tobacco, *coxIV* presequence from yeast, *orfH522* gene from sunflower (responsible for the *PET1* CMS) and the *nos*

terminator from *Agrobacterium tumefaciens* (Harinath, 2004). As the orientation of all the components had already been confirmed by restriction analysis, PCR and sequencing, the strategy for development of sterility induction vectors using unedited versions of *nad3/atp9* (*u-nad3/u-atp9*) mitochondrial genes from safflower involved replacing the *orfH522* gene in TCON with the *u-nad3/u-atp9* genes. To achieve this, primers were designed with appropriate restriction enzymes that were used for cloning *orfH522* in the cassette. This would facilitate facile replacement of *orfH522* with *u-atp9/u-nad3* genes. These enzymes did not have any internal sites within *u-nad3/u-atp9* and thus could be used without any serious consequences either at the time of cloning and further analysis.

The confirmed genomic T/A clones for *nad3* and *atp9* genes which had the PCR product resultant from the F2 and R2 set of primers (that included only the coding portion of these genes and not the flanking regions) were used. The orientation of the inserts in the T/A vector was first confirmed by restriction digestion and clones that yielded the desired fragment (to be cloned) only when both the cloning enzymes were used for digestion and not when only one of them was used, were selected for the purpose of downstream cloning. This ensured that the fragments had the required enzyme sites at the end, which would clone these fragment in right orientation. Otherwise, it could be conjectured that if the fragment had resulted from the action of a single enzyme (having one site in the PCR product and the other from the MCS of the vector), the fragment can get cloned in wrong orientation in the vector that is also digested only with that particular enzyme.

The genomic fragments that represent the unedited versions of the genes, were cloned in place of *orfH522* in the pCAMBIA1305.2-TCON vector, to generate pCAMBIA1305.2-TCNN and pCAMBIA1305.2-TCAN, respectively. Though restriction

with the enzymes flanking the *nad3* and *atp9* genes, confirmed their presence by giving 357 bp and 261 bp, respectively, serial PCR confirmed the presence as well as orientation of all the components of the cassette. Here, one forward primer which binds to the 5' end of the construct was kept common and the reverse primers of each of the succeeding component were used for amplification in a serial order. This generated expected amplicons in increasing order of sizes that confirmed their presence as well as appropriate orientation within the cassette (Figure 4.25).

From these confirmed vectors, the entire cassettes of 'TCNN' and 'TCAN' were restricted out by using *HindIII* and *EcoRI* and cloned into pCAMBIA0390. The binary vector pCAMBIA0390 was used for this purpose, as it did not have any plant selection marker and the *bar* gene cassette could be cloned into it. As discussed earlier, the T-DNA should preferably contain a plant selection marker that could be used at the plant level in the field to select the sterile transgenic plants in the segregating population obtained by crossing the male sterile with the corresponding non-transgenic plants. Therefore, it was intended to clone the 'bar' gene cassette that confers resistance to the herbicide phosphinothricin, so that the same selection pressure could be used for both *in vitro* and field selection.

With this idea, the *bar* gene cassette was cloned into the pCAMBIA0390-TCNN and pCAMBIA039-TCAN vectors. The *bar* gene cassette was obtained from the pBARGUS plasmid kindly supplied by Prof. Rongdu of NCSU, USA, as a *HindIII* digested fragment. In this cassette, the *bar* gene is under the control of 35S promoter and with polyA transcriptional termination signal.

The orientation of the *bar* gene with respect to the male sterility inducing construct is very important, more so because the *bar* gene is driven by the constitutive 35S promoter

whereas the TCNN and TCAN constructs are under the tapetal specific TA29 promoter. The CaMV 35S is a strong constitutive promoter and it has been reported that its use to drive the marker gene could result in leaky expression of the adjoining male sterility causing gene resulting in abnormalities such as poor female fertility, low seed germination frequencies and/or distortion in segregation ratios of transgenes (Jagannath *et al.*, 2001). Hence, in the present study, orientation of the *bar* gene cassette was determined by restriction analysis (Plate 4.28). The clones which had the *bar* gene cassette and the sterility inducing cassette (TCNN/TCAN) cloned in opposite orientations were used for transformation work.

Transgenic male sterility has been induced using unedited mitochondrial *atp9* and *nad9* genes. Male sterile rice plants have been obtained using *u-nad9* gene fused to the coding sequence of *Arabidopsis* mitochondrial transit peptide (At-mRBP1a) under the control of ubiquitin promoter (http://www.avesthagen.com/docs/newsletters/jan_feb_2005.pdf). Hernould *et al.*, 1993 have expressed *u-atp9* (from wheat) under CaMV35S promoter along with *coxIV* presequence and obtained transgenic male sterile tobacco plants. Similar gene cassettes using ap3 and A9 tapetal specific promoters and the constitutive CaMV 35S promoter were used to transform *Arabidopsis*. But only the constructs with tapetal specific promoters were successful in inducing male sterility in *Arabidopsis* (Gomez-Casati *et al.*, 2002). In the present study too, as mentioned above, the tapetal specific TA29 promoter and *coxIV* presequence (whose efficiency has been proved), were utilized for development of constructs.

5.5 Development of constructs for restoration of fertility

Presence of fertility restorer lines that can restore complete fertility in the male sterile lines are a prerequisite for development of an efficient male sterility-restoration system. This holds good also for a transgenic system where male sterility is due to a dominant mutation. The restorer lines must be capable of abolishing the functionality of protein encoded by the male sterility causing transgene. Literature survey shows that though there are abundant studies on induction of transgenic male sterility, reports on transgenic fertility restoration are very few.

Restoration could be effected at DNA level, RNA level and protein level. Restoration at the DNA level could be achieved by elimination of the male sterility-inducing cassette from the genome. Strategies for this have been developed by exploiting the site specific recombination systems such as FLP-FRT, CRE-LOX taken from 2 μ m plasmid of yeast and bacteriophage, respectively (Luo *et al.*, 2000; Bayer and Hess, 2005). The best example for restoration at protein level is the barnase-barstar system where the inhibition of the barnase (ribonuclease) protein by a corresponding barstar (ribonuclease inhibitor) protein leads to fertility restoration of the transgenic male sterile lines. Restoration at RNA level is effected when the target transcripts (which code for the male sterility causing protein) are eliminated or the translation process is inhibited. The silencing at the RNA level by antisense strategy has been widely used.

Majority of the studies utilize the antisense versions of the male sterility causing transgenes to develop constructs for fertility restoration (Zabaleta *et al.*, 1996; Busi *et al.*, 2006; Hird *et al.*, 2000). Expression of such antisense molecules in the hybrid plants lead to degradation of the male sterility causing transgene transcripts leading to lack of

corresponding protein and thereby giving normal fertile plants. But antisense approach is shown to be less efficient in transgene silencing which necessitates the production of a large number of independent transgenics in order to select the appropriate plant that can bring about fertility restoration. This would be a major limitation especially in species such as safflower, where the transformation and regeneration are still a problem.

Though there are more efficient DNA directed RNA interference (ddRNAi) technologies such as intron-interrupted hairpin RNA (ihp-RNA) and SHUTR (Silencing by heterologous 3' untranslated region) for silencing the target genes, surprisingly these approaches have not been adopted for silencing the male sterility inducing genes. In the present investigation, both antisense as well as ihp-RNA approaches were intended to be compared for their ability to restore fertility. Therefore, both types of constructs were developed against *nad3* and *atp9* genes. However, due to lack of efficient protocols for regeneration and transformation in safflower and to reduce the workload to meaningfully achievable targets, transformation was attempted only with vectors that effect silencing through intron interrupted hairpin RNA (ihp-RNA).

The vectors for fertility restoration were initially made under 35S promoter. However, it has been reported that 35S promoter has a low expression in the floral organs especially in the anther tapetal tissues (Duroc *et al.*, 2006). In approaches where tapetal specific promoters have been used for induction of male sterility, the restoration gene constructs also are under the same promoter (Mariani *et al.*, 1992; Busi *et al.*, 2006). Such a strategy of using the same promoter for restoration constructs would increase the efficiency of gene silencing, thereby giving more efficient restoration of fertility. Since in our approach, constructs for induction of male sterility are under TA29 promoter, the restoration

constructs too under the same promoter would increase the efficiency of gene silencing, thereby giving more efficient restoration of fertility. Hence, it was considered logical and prudent to develop the fertility restorer constructs with TA29 promoter.

For the development of the full-length antisense constructs and *ihp*-RNA constructs, the pRT100 was used as the basal vector. The choice of this vector was most appropriate as it had 35S promoter and the polyA terminator with MCS in between into which the genes were cloned in the antisense orientation using appropriate restriction enzymes (development of p-SANP and p-SAAP) (Figure 3.11). The 35S promoter was then replaced with the TA29 promoter (from the T/A-TA29 clones) in order to generate the p-TAAP and pTANP vectors (Figure 3.13).

At the Directorate of Oilseeds Research, Hyderabad, generic vectors for gene silencing viz., Intron-interrupted Hairpin RNA (IHP) and Silencing of Heterologous Untranslated Region (SHUTR) had been developed in pRT100 vector background (Ashraf *et al.*, 2007). Using these vector systems, the *ihp nad3/ihp atp9* constructs were developed by cloning the component sequences (sense, antisense gene segments separated by a functional catalase intron) between the 35S promoter and polyA signal. For this purpose, appropriate enzymes were chosen to facilitate cloning of all the components in the right orientation (Figure 3.12).

During development of *ihp*-RNA constructs under TA29 promoter, it was not possible to directly replace the 35S promoter by the TA29 promoter as done during development of p-TANP and p-TANP clones. This was because though *HincII* and *XhoI* enzymes flanked the 35S promoter in *ihp nad3* and *ihp atp9* constructs, these had an additional *XhoI* site at the downstream end of the sense *u-nad3/u-atp9* genes (which was

included in the reverse primer and was used during cloning of the sense-genes). Due to this, restriction with *HincII* and *XhoI* would separate out the 35S promoter as well as the ihp fragments from the background vector (pRT100). To overcome this problem, another strategy was followed (Figure 3.14). The TA 29 promoter was first cloned into the pRT100 vector using *HincII* and *XhoI* enzymes. After confirmation of this p-TA29 clone, then the ihp fragment alone (antisense *u-nad3/atp9*-cat int-sense *nad3/atp9*, obtained by restricting the p-ihp *nad3/p-ihp atp9* clones) was cloned downstream of the TA29 promoter using *XhoI* enzyme into this vector (Figure 3.14). Thus the p-T-ihp *nad3* and p-T-ihp *atp9* clones were developed. The orientation of the open reading frame with respect to the TA29 promoter was confirmed by PCR and restriction analysis.

All the confirmed restoration cassettes developed under pRT100 backbone, were cloned into the binary vector pCambia1300 and confirmed for presence and orientation of all the components.

5.6 Mobilization of the recombinant binary vectors into *Agrobacterium*

Confirmed clones of each of the recombinant binary vectors were mobilized into *Agrobacterium* strain LBA4404 using electroporation method. *Agrobacterium*-mediated plant transformation system offers unique advantages due to its simplicity and precise transfer of DNA sequences with defined ends making it a “poor man’s vector” (Veluthambi *et al.*, 2003). This system is of much importance due to its higher frequency of stable transformation with single copy insertions. Hence, this is the most preferred method among several transformation methods available. All the binary vectors were transferred into *Agrobacterium* strain LBA4404 through electroporation as the transfer rate is high unlike freeze thaw method where the transfer rate is low. Also this occurs more in triparental

mating method of transfer of recombinant DNA into *Agrobacterium*. Mobilization of binary vectors into *Agrobacterium* and its presence was confirmed by restriction analysis and PCR amplification of the plasmids.

The plasmid DNA from *Agrobacterium* clones were also subjected to PCR and/or restriction analysis. As the binary vectors are maintained in *Agrobacterium* strain as low copy plasmids, the yield of the plasmid DNA obtained from *Agrobacterium* strain was always low and most of the times it was difficult to carry out restriction analyses. To circumvent this problem, chemically competent *E. coli* cells were transformed with the plasmid DNA isolated from *Agrobacterium* (reverse mating) and the plasmid DNA isolated from *E. coli* colonies obtained was confirmed by colony PCR/gene specific PCR and restriction analyses to ensure that the plasmid DNA had not undergone any recombination during the process of electroporation. One each of the confirmed *E. coli* clones were also sequenced and analyzed so that there were no mutations in the sequence. Only those *Agrobacterium* clones, thus thoroughly confirmed, were used for transforming safflower and tobacco.

5.7 Tissue culture of safflower

Attempts were made in the present investigation towards shoot regeneration in safflower *via* the embryogenetic pathway. It was observed that culture of immature embryos on any of the three primary media combinations MS + 3% sucrose + TDZ 1.0 mg/l + NAA 0.2 mg/l (SV1), MS + 3% sucrose + TDZ 0.5 mg/l + NAA 0.2 mg/l (SV2) or MS + 3% sucrose + TDZ 0.5 mg/l + NAA 1.0 mg/l (E1) and then transfer of the responding explants into secondary medium BA 0.2 mg/l gave rise to adventitious shoots in higher frequency.

Similar kind of shoot regeneration from immature embryos was obtained with different combinations of TDZ and NAA (Orlikowska and Dyer, 1993) whereas direct somatic embryos were induced from cotyledonary leaves using BA + NAA and these were germinated (in medium with NAA) to obtain complete plants successfully (Mandal *et al.*, 1995). Despite success in these reports and in the present study, direct regeneration from seedling explants is superior over the use of immature embryos because of seasonal independence of availability of a large number of explants, easier explant preparation and the better vigor of regenerated shoots (Orlikowska and Dyer, 1993). Also there are reports that the embryogenetic pathway of regeneration may not be advantageous for transformation as the probability of the embryogenic cell getting transformed is rather low given the lower frequency of the embryogenic cells in the explants.

Though attempts have been made in safflower towards shoot induction, root induction and transformation independently, there is no single efficient protocol to achieve efficient regeneration coupled with transformation. Among all the basal media tried, Murashige and Skoog medium (Murashige and Skoog, 1962) has been proved to be superior for induction of morphogenetic response in both somatic and gametic tissues (Prasad *et al.*, 1991; Chatterjee and Singh, 1993). This basal medium was used for all media combinations during tissue culture and transformation in the present investigation.

Investigators have attempted using different media combinations for shoot regeneration in safflower through organogenic and embryogenic pathways. These include successful use of: BA singly (Mandal and Gupta 2001; Nikam and Shitole, 1999); BA in combination with NAA (George and Rao, 1982; Anwar *et al.*, 1989; Singh, 1991; Prasad *et al.*, 1991, Chatterjee and Singh, 1993, Orlikowska and Dyer, 1993; Zhanming and Biwen,

1993; Sujatha and Suganya, 1996; Nikam and Shitole, 1999); BA in combination with kinetin (Vijayakumar and Kumari, 2005); and TDZ with NAA (Orlikowska and Dyer, 1993; Mandal *et al.*, 1995; Radhika *et al.*, 2006). Kinetin, 2-isopentyladenine and zeatin singly and in combination have also been used but with no success (George and Rao, 1982). Of all these, the TDZ + NAA combination was found to be the best in terms of frequency of responding explants, response from all tissue types and tested genotypes and number of shoots per responding explant (Orlikowska and Dyer, 1993; Radhika *et al.*, 2006). At the biotechnology laboratory, DOR, two media combinations MS + TDZ 1.0 mg/l + NAA 0.2 mg/l (SV1) and MS + TDZ 0.5 mg/l + NAA 0.2 mg/l (SV17), were identified as best for shoot induction. These combinations were used for the experiments under the present investigation.

Attempts have also been made towards enhancing the frequency of root induction in regenerated shoots. These include use of: hormone-free basal MS medium (Anwar *et al.*, 1989; Rani and Rao, 1998; Chatterjee and Singh, 1993); 2,4,5-trichlorophenoxy propionic acid (0.5-1.0 mg/l) alone or in combination with BAP or TIBA (0.5 mg/l) (Anwar *et al.*, 1989; Chatterjee and Singh, 1993; Baker and Dyer, 1996); NAA alone (Rani and Rao, 1998; Sujatha and Suganya, 1996) or in combination with kinetin (Zhanming and Biwen, 1993). In the present investigation half-strength MS medium with NAA 0.5 mg/l (RM2) was tried for root induction based on an earlier report (Sujatha and Suganya, 1996) and was found appropriate. Also at the laboratory in DOR, another combination involving IBA 2.0 mg/l and phloroglucinol 2.0 mg/l (RM₁) (Rao *et al.*, 2007), was found to be optimal for root induction. Both these rooting media (RM₁ and RM₂) were used in the present investigation and it was found that frequency of root induction was higher in RM₂ than RM₁. The former

showed a cluster of thick roots whereas the roots obtained in the latter were longer and thread-like.

Another challenging aspect of safflower tissue culture is the acclimatization and survival of rooted shoots. There are very few attempts on these aspects in safflower. Orlikowska and Dyer (1993) reported an 80% survival rate in the transfer of 4 cm long rooted safflower shoots to soil. However, shoots that were not sufficiently elongated did not show better survival. Nikam and Shitole, 1999 reported a 34% success rate in transfer of rooted shoots to soil and stressed the need for further improvements in the regeneration efficiency and survival of plantlets when transferred to the field as these aspects vary with the cultivar used. In the present investigation, mortality during acclimatization was the major bottleneck especially with shoots that were not elongated atleast upto 3-4 nodes. Though more than 60% of the rooted shoots died even when they were being acclimatized in sterile vermiculite pots in the tissue culture lab, more than 80% of those which survived under lab conditions, were lost 3-20 days after transfer to larger pots (with soil and FYM) in the green house. This became the major bottleneck in transferring the transformed shoots to soil and making them survive.

The vast difference in post acclimatization survival rates of safflower in the above reports and the severely reduced survival rate in our study could not be deciphered. However, further work is needed in these aspects to improve the post-acclimatization survival rate of the regenerated safflower plants.

5.8 Transformation of safflower

A simple protocol was standardized at the Biotechnology Laboratory, Directorate of Oilseeds Research, for transformation and regeneration using seedling explants derived from

8-10-days-old aseptic seedlings, which was used for transformation studies in this investigation. The transformation frequency was lower with root explants than with hypocotyls explants. But the quality of the shoots arising from both these explants were good with the exception of a few shoots rarely appearing to be vitrified (glassy or water soaked appearance) and also for those shoots which were maintained on the shoot multiplication/elongation medium for more than 3-4 cycles. Lowering cytokinin concentration (Leshem *et al.*, 1988; Paques and Boxus, 1987) or substituting one cytokinin for another (usually replacing BA with a different cytokinin) has been reported to be useful to avoid vitrification (or hyperhydricity). In the present investigation too, initially, some safflower transgenic shoots were lost due to hyperhydricity especially noticed in shoots that were subjected to prolonged culture on media with BA (0.5 mg/l). But subsequently, shoots from selection III were transferred to media with a lower BA (0.2 mg/l) concentration or to kinetin 0.5 mg/l (different cytokinin) and this helped in avoiding hyperhydricity. But the shoots that already appeared severely vitrified (whole shoot giving a watery appearance) did not recover on any of the media tried. Also it was noted that shoots under prolonged subculture lost their healthy appearance and did not respond for root induction.

Successful *Agrobacterium* mediated transformation of safflower calli as well as seedling explants and the factors influencing the same have been reported (Orlikowska *et al.*, 1995) which has proved that safflower is amenable to *Agrobacterium* mediated genetic transformation. But the problems in safflower tissue culture were further aggravated in genetic transformation experiments where the regenerated shoots were subjected to exposure to the bacteriostats and selective genes like antibiotics or herbicides (reviewed by Sujatha, 2007). In the present study, though transformation frequencies varied with different

constructs, there were no problems in the transformation procedure *per se* or the selection of the transformants except for minor hindrances such as vitrification of some shoots, loss of shoots due to contamination *etc.* But the major problems were with respect to elongation of shoots to sufficient length (atleast 4 nodes), lack of response in shoots subcultured for a prolonged time, rooting of the shoots, acclimatization of shoots and their survival upon transfer to green house. Though the problems with rooting as well as to some extent elongation could be overcome, the post-acclimatization survival was the major bottleneck due to which only a very small number of transgenic plants could reach the flowering stage (Table 4.10).

5.9 Transformation of tobacco

Though the target crop for our investigation was safflower, it was decided to transform tobacco also simultaneously with the four selected constructs. This was undertaken as tobacco, being a model system for regeneration and transformation studies, would facilitate easier and quicker validation of the developed constructs. A perusal of literature on transgenic male sterility indicates that most of the attempts at induction of male sterility have been in the model crops tobacco and *Arabidopsis* (Table 3.2). The most widely used and successful transgenic male sterility-fertility restoration system, the *barnase-barstar* system, was first successfully attempted in tobacco after which it was transferred to oilseed rape (Mariani *et al.*, 1990) and later in other crops like cauliflower, tomato, cotton, corn, wheat, chicory *etc.* (Banga *et al.*, 2006). In the present study, as expected, tobacco responded well in terms of transformation frequency, elongation of transformed shoots, root

induction and also, all the plants (with each of the constructs) that were transferred to green house survived and flowered without any technical problems.

Though more number of TCNN-Bar tobacco shoots were produced and confirmed under the present investigation, the number of tobacco shoots produced for the other three constructs were fewer (Table 4.12). Hence, as a future line of work, more number of tobacco transgenic plants need to be produced with these constructs so that the performance of their expression can be fully studied.

5.10 Analysis of the transgenic plants

In the present study, all the TCNN-Bar transgenic tobacco plants obtained were similar to the untransformed control plants with respect to morphological traits such as plant height, number of leaves, number of flowers, *etc.* Similar results have been reported in most of the studies on transgenic male sterility, where the transgenic plants (either completely male sterile or semi-sterile or fertile) were similar to control (untransformed) plants in their vegetative morphology indicating that the action of the transgene as well as the promoter were highly specific to the target tissues. However He *et al.*, 1996 observed retarded vegetative growth when *orf239* from common bean was expressed in tobacco under both constitutive (sp) promoter and pollen specific (Lat52) promoter. Another interesting observation was that when *u-atp9* from wheat was expressed in tobacco under constitutive promoter, no changes in the vegetative morphology were seen in the transgenic plants (Hernould *et al.*, 1993), but the *Arabidopsis* transgenic plants (harbouring the same construct) exhibited reduction in plant height, number of leaves and number of flowers (Gomez Casati *et al.*, 2002). In the same study, when the construct was also expressed under

anther specific promoters A9 and ap3, it led to complete as well as semi sterile transgenic plants (Gomez Casati *et al.*, 2002). In the present investigation, seven out of the 30 TCNN-Bar transgenic tobacco plants showed partial sterility (10 to 76.8% pollen fertility). There were variations among these plants with respect to the position of stigma and anthers. While three plants showed protruded stigma with regressed anthers that were far below the level of stigma, some had a mixture of such flowers as well as normal flowers and the remaining plants showed normal floral morphology (stigma at the same or slightly below the level of anthers).

Flower drop was common in all the partial sterile as well as some fertile plants. All the plants set seeds but all the partial sterile plants as well as some fertile plants had either only small sized capsules or both normal and small capsules. One male sterile plant (no 99-1) in particular, that had showed less than 5% darkly stained pollen (with acetocarmine), exhibited very severe flower drop and did not form capsules. However, capsules with seeds were obtained when the flowers were pollinated manually with the pollen from untransformed plant. This clearly indicated the normal female fertility of this plant.

Though these observations were for the tobacco transgenic plants, the same could not be corroborated in safflower due to the small number of flowered transgenics obtained in the present study. However it was observed that the transgenic safflower plants were weaker and lanky in appearance and the flowers were very small with very few florets compared to the control untransformed plants. Though we observed partial sterility in three of six TCNN-Bar, and one of the five TCAN-Bar flowered transgenic plants, these numbers are very small to speculate the action of these transgenes in safflower. However, the results obtained indicate that male sterile plants could be obtained in safflower, if more number of

independent transgenic plants are developed and studied at the flowering stage. Hence, rectifying the problems in the safflower tissue culture and post acclimatization process faced during the present study should form the future line of work.

Similar to the results obtained in the present investigation, there are many reports on induction of transgenic male sterility where only a few completely male sterile and/or partial sterile plants were seen out of a large number of plants obtained (He *et al.*, 1996; Duroc *et al.*, 2006; Zhan *et al.*, 1996; Goetz *et al.*, 2001). The reason for this could be the incomplete penetrance or insufficient expression at the critical stage or the inability of the promoter to direct expression at the required stage (He *et al.*, 1996). Another reason could be accumulation of too little of protein or inappropriate folding or submitochondrial location of the protein. This can be proved only by further quantitative analysis. In the present study, the expression levels of the introduced transgenes have not been estimated. Thus it can only be speculated that the expression levels might not have been sufficient to induce the expected phenotype (male sterility). Duroc *et al.*, (2006), have found that all of the transgene-encoded protein may not be targeted to the correct submitochondrial location that may lead to absence of complete male sterile plants. However these reasons might not stand in our case, as the tapetal specific expression pattern of TA29 promoter and the mitochondrial targeting by the *coxIV* presequence, which have been used in the present study have already been proven in safflower (Kalyani *et al.*, 2007)

Also, in the present investigation, the choice of TA29 promoter for *atp9* is appropriate as it is already reported that the expression of *u-atp9* affects the tapetal tissue by causing hypertrophy, high degree of vacuolization and premature degeneration in these cells (Hernould *et al.*, 1998). Since TA29 promoter is specific to tapetal cells, the expression of

the unedited version in these cells seems to be most appropriate. Though *nad3* was being used for the first time for induction of transgenic male sterility (in the present investigation), since it codes for a subunit of an important mitochondrial protein complex (NADH oxidoreductase), we hypothesized that the expression of the *u-nad3* in the tapetal tissues could lead to male sterility. However, only plants with reduced fertility have been obtained, which proves that expression of this gene must be leading to disturbance in normal mitochondrial functioning.

It can also be conjectured that even if the activity of the promoter and transgene are coordinated, there could be correlation of the dosage of the transgene and the level of reduction in male fertility. In many studies it has been seen that more copy number of transgene leads to complete male sterility whereas reduced/single copy could lead to leaky phenotype or partial fertility (Zu *et al.*, 1995; Hernould *et al.*, 1993; Zhan *et al.*, 1996). Since under the present investigation, Southern blotting has not been attempted, the information on the transgene copy number was not generated. But if more number of plants are developed and studied, such a correlation, if present between copy number and degree of fertility, could be detected.

Another reasoning that could be considered is that the U-NAD3 protein is not cytotoxic and hence is not expected to destroy the target cells. It is only expected to compete with the native NAD3 protein in the assembly of the NADH oxidoreductase complex, so that it would impair the mitochondrial functioning. Hence a higher quantitative level of this U-NAD3 protein might be required to successfully outweigh or counter the activity of the native protein. This could lead to depleted energy production in the mitochondria in tapetal cell layer leading to disruption in microsporogenesis.

Transgenic plants are usually confirmed by PCR analysis for the presence of the transgene, morphological observations including the trait of interest, Southern blotting for the copy number/orientation of the insert(s), northern blotting for the expression of the transgene, assays for sensitivity to herbicide/antibiotic/other compounds (or histochemical assays) as per the selection gene(s) present in the insert, cytological analysis (where pollen fertility/have to be determined) *etc.*

In the present investigation, the developed safflower and tobacco transgenic plants were subjected to morphological observations, PCR analysis, leaf assays for phosphinothricin/hygromycin sensitivity and pollen analysis (pollen fertility including pollen germination analysis for some of the transgenics). PCR was done using appropriate combination of primers so as to confirm the presence of the entire transgene.

Initially the PCR analyses were performed for those shoots in the maintenance/elongation medium, from which sufficient leaf sample could be collected. During this analysis, it was found that only some of the shoots actually carried the transgene (safflower shoots TCNN-Bar: 25/80, TCAN-Bar: 13/65, tobacco shoots TCNN-Bar: 41/70, TCAN-Bar: 4/17) indicating presence of a large number of escapes in both safflower and tobacco (Table 4.12). This meant that the selection regime of phosphinothricin 0.5 mg/l (S I), 1.0 mg/l (SII), 1.0 mg/l (SII) and hygromycin 10 mg/l (S I), 15 mg/l (SII), 20 mg/l, (SIII) used in the present study may not be efficient in selecting the transformed explants/shoots and has to be altered so as to avoid escapes and select only the transgene harbouring plants. However, the selection regime used in this study was developed after detailed experiments were conducted on the sensitivity of safflower explants to hygromycin and phosphinothricin (Rao *et al.*, 2007). Also along with transformed explants, the control (untransformed)

explants had also been subjected to the same selection pressure and ~95% of these explants were found to be killed due to effect of phosphinothricin. The reason behind these contradictions needs to be investigated and rectified by trying some more modification in the selection regime. But all the plants that exhibited sterility (during pollen fertility analysis), were positive in the PCR analysis and also during the phosphinothricin/hygromycin assays. However, it should be noted that the plants were analysed for PCR with respect to the gene of interest and not for selectable markers. As rearrangements in T-DNA are very common in transgenics, it cannot be ruled out that during integration of the T-DNA, some plants might not carry gene cassette of our interest but could carry only the gene cassette for the selection marker. Such plants would be obviously negative for PCR with transgene specific primers. In the present study, this possibility has not been ruled out. The pollen from the sterile tobacco plants were observed for their *in-vitro* germination and showed severe abnormalities during growth in comparison to the control pollen. The few pollen in the male sterile TCNN-Bar plant (99-1), did not germinate at all.

Though leaf assays and PCR analysis did confirm the transgenic status of these plants, further confirmatory analyses like Southern blotting to determine the transgene copy number, northern and/or western blotting to confirm the expression of the transgene need to be done to further confirm the transgene and its expression. Though partial sterile lines in safflower and both complete as well as partial sterile tobacco plants have been developed with the *u-nad3* constructs, these must be carried forward to the next generation (T_1) and analyzed for the stable integration and expression of the transgenes. Also appropriate homozygous plants with the restoration constructs must also be selected at the T_1 generation and must be used for testing the fertility restoration. In case of safflower, further

improvements to the transformation and regeneration protocol as well as hardening process, are necessary to realize more number of healthy transgenic shoots that can be analyzed efficiently and carried forward to the next generation. All these shall form the future line of work.

In conclusion, the positive aspects of this study are that this is the first time report of RNA editing in the mitochondrial genes of safflower. A preliminary proof-of-concept has been obtained that the *u-nad3* as a candidate gene could lead to male sterility. Also the partial sterile plants obtained in safflower and tobacco and the male sterile plant obtained in tobacco indicate that the expression of the *nad3* gene is important for the normal mitochondrial functioning. Though *u-atp9* gene (from wheat) has been used in a similar attempt, the *u-nad3* gene has been used for the first time in this study for induction of male sterility. The constructs developed in this study as well as the transgenic plants could form the base material towards realizing a good transgenic male sterility-fertility restoration system in safflower as well as other heterologous plants.

CHAPTER VI

SUMMARY

CHAPTER VI

SUMMARY

The present investigation was taken up with the aim of developing transgenic male sterile and restorer lines in safflower. The hypothesis that RNA editing of mitochondrial genes is crucial for the proper functioning of mitochondria, was used as the basis of this study. It was intended that if unedited version of important mitochondrial gene(s) is expressed under tapetal tissue specific promoter, and the resultant protein is targeted into mitochondria, this could impair proper functioning of the mitochondria leading to male sterility.

For identification of the appropriate candidate genes for this approach, three mitochondrial genes viz., *atp6*, *atp9* and *nad3*, were studied. Primers for these genes were designed based on the corresponding sunflower sequences and used to amplify the genes in safflower. PCR and RT-PCR were done to develop the genomic and cDNA clones for these genes and analyze the RNA edited sites by comparing the nucleotide sequences of the clones. The RNA editing of safflower *atp6* could not be studied as the full-length cDNA sequence could not be obtained but it was found that in sunflower *atp6* transcripts undergo RNA editing at only two sites. *nad3* and *atp9* gene transcripts of safflower showed 19 and 12 RNA editing sites respectively and analysis of their deduced proteins indicated that editing increased the hydrophobicity of these proteins. These results indicated the importance of these genes as well as RNA editing of their transcripts that made them appropriate candidates for this approach.

To attempt the possibility of regenerating shoots in safflower using embryogenetic pathway, immature embryos were cultured on media supplemented with different combinations of growth hormones. Eighteen primary media combinations and five secondary

media combinations were tried. Among these different combinations of media tried, culturing on MS + 3% sucrose + TDZ 1.0 + NAA 0.2 mg/l (SV₁), MS + 3% sucrose + TDZ 0.5 + NAA 0.2 mg/l (SV₂) or MS + 3% sucrose + TDZ 0.5 mg/l + NAA 1.0 mg/l (E₁) as primary media and the transfer of the responding explants into secondary medium BA 0.2 gave rise to adventitious shoots in higher frequency. Though a low frequency of somatic embryos were formed, a higher frequency of adventitious shoots were obtained in the present study.

For the development of constructs for induction of male sterility, *u-nad3* and *u-atp9* genes were cloned in place of *orfH522* gene in the pCAMBIA1305.2-TCON vector developed at the biotechnology laboratory, DOR, using *KpnI* and *SacI* enzymes to generate pCAMBIA1305.2-TCNN and pCAMBIA1305.2-TCAN. The entire cassettes of TCNN and TCAN were then excised out using *HindIII* and *EcoRI* and cloned into pCAMBIA0390 to obtain pCAMBIA0390-TCNN and pCAMBIA0390-TCAN. The entire bar gene cassette (bar gene under the control of 35S promoter) from the pBARGUS vector was cloned as a *HindIII* fragment in pCAMBIA0390-TCNN and pCAMBIA0390-TCAN, which resulted in plant transformation vectors, the pBin-TCNN-Bar and pBin-TCAN-Bar respectively.

For restoration of fertility, the full-length antisense as well as ihp-RNA constructs were developed initially in the pRT100 vector background and later the gene cassettes were cloned in the binary vector pCAMBIA1300. The *nad3* and *atp9* genes were cloned into the pRT100 vector using *KpnI* and *SacI* enzymes, which ensured the antisense orientation of the genes and resulted in constructs pSANP and pSAAP. The 35S promoter in these constructs was then replaced by TA29 promoter using the *HincII* and *XhoI* enzymes (pTANP and pTAAP). The entire SANP, SAAP, TANP and TAAP cassettes were then cloned into the

binary vector pCAMBIA1300 using *Hind*III enzyme (pBin-SANP, pBin-SAAP, pBin-TANP and pBin-TAAP).

For development of the ihp-RNA constructs, the pRT100-Cat Int (pRT100 vector developed earlier at DOR with a functional catalase intron from castor cloned in the MCS) was used as the background vector. Initially the genes were cloned into pRT100-Cat Int in the antisense orientation using *Sac*I and *Xho*I enzymes. Then the *nad3* and *atp9* genes were cloned in the sense orientation using *Kpn*I and *Bam*HI enzymes to generate p-ihp *nad3* and p-ihp *atp9* clones. The entire cassettes i.e., CaMV35S promoter-ihp *nad3*-polyA terminator and CaMV35S promoter-ihp *atp9*-polyA terminator, were excised out using *Hind*III enzyme, and were cloned into *Hind*III digested pCAMBIA1300 to realize pBin-ihp *nad3* and pBin-ihp *atp9* vectors. For development of these constructs under TA29 promoter, initially pRT100-TA29 was developed using *Hinc*II and *Xho*I enzymes that replaced 35S promoter with TA29. Into this clone, the entire ihp *nad3* and ihp *atp9* cassettes were cloned using the *Xho*I enzyme so that p-T-ihp *nad3* and p-T-ihp *atp9* were obtained. From these vectors, the T-ihp *nad3* and T-ihp *atp9* cassettes were cloned into pCAMBIA1300 using *Pst*I enzyme to obtain pBin-T-ihp *nad3* and pBin-T-ihp *atp9* clones.

All the constructs were confirmed thoroughly by PCR and/or restriction digestion for the presence and the orientation of the components. These were then mobilized into *Agrobacterium* strain LBA4404 and were further confirmed. Four of these vectors, two constructs for induction of male sterility i.e., LBA4404: pBin-TCNN-Bar and LBA4404: pBin-TCAN-Bar as well two constructs for restoration of fertility i.e., LBA4404: pBin-T-ihp *nad3* and LBA4404: pBin-T-ihp *atp9* were used to transform safflower and tobacco.

Transgenic safflower and tobacco plants were developed with all the four constructs. All the transgenic plants were confirmed by PCR analysis for the presence of the transgene and by leaf assays for sensitivity to phosphinothricin or hygromycin (depending on the vector used). The total number of safflower transgenic shoots obtained ranged from 35 to 161 where as the number of rooted shoots ranged from 3 to 53 for different constructs. These transgenic safflower shoots were twiny as well as lanky in appearance and had smaller flower buds with less number of florets. Among the plants that reached flowering stage, three out of the six TCNN-Bar and one out of five TCAN-Bar transgenic plants, showed partial sterility (34.2%-69% and 32% pollen fertility respectively).

Of all the tobacco transgenic shoots that reached flowering stage, one TCNN-Bar plants showed male sterility with no seed set-upon selfing and seven plants showed partial sterility (22% to 76.8% pollen fertility). Pollen germination studies revealed that none of the few pollen in the male sterile plant germinated and the partial sterile plants showed several abnormalities in the pollen tube germination. Though morphological appearance of the tobacco transgenic plants was similar to control untransformed plants, there were differences in the floral morphology of some plants. The male sterile as well as partial sterile transgenic plants had smaller sized flower buds with some plants showing highly protruded stigma with anthers regressed below the stigma (whereas in the control as well as other fertile transgenic plants, anthers were always either above stigma or at the same level as stigma). The single male sterile plant (99-1) showed severe flower drop and could set capsules only upon manual pollination with pollen from untransformed plant indicating the normal female fertility in this plant.

The results of the present investigation have provided the groundwork and preliminary indications of proof-of-concept that these constructs based on *u-nad3* and *u-atp9* genes, could lead to male sterility in safflower and tobacco. As a future line of work, more number of transgenic plants will have to be generated with the constructs to further validate them, which eventually is expected to develop into a complete pollination control system, not only in safflower, but also in other crops.

LITERATURE CITED

LITERATURE CITED

- Araya A Zabaleta E Blanc V Begu D Hernould M Mouras A and Litvak S 1998 RNA editing in plant mitochondria, cytoplasmic male sterility and breeding. *Electronic Journal of Biotechnology* 1 : 1-14.
- Ausubel F M Brent R Kingston R E Moore D D Seidman J G Smith A and Streehl K 1999 *Short Protocols in Molecular Biology*, 4th edition. John Willey and Sons, Inc., New York.
- Abad A R Mehrtens B J and Mackenzie S A 1995 Specific expression in reproductive tissues and fate of a mitochondrial sterility associated protein in cytoplasmic male sterile bean. *Plant Cell* 7 : 271-285.
- Akagi H Sakamoto M Shinjyo C Shimada H and Fujimura T 1994 A unique sequence located down stream from rice mitochondrial *atp6* may cause male sterility. *Current Genetics* 25 : 52-58.
- Anjani K 1997 Safflower hybrids developed using recessive genetic male sterility system. *Proceedings IV International Safflower Conference, Bari, Italy*, 281-282.
- Anjani K 1998 DSH 129: The first safflower hybrid released for commercial cultivation. In: *Proceedings National Seminar on Heterosis: Its Commercial Exploitation*. Nagpur, India, 32.
- Anwar S Y Khadeer M A and Tejovati G 1989 Genetic and tissue culture studies in safflower. *Recent Advances in Genetics and Cytogenetics*, (Eds. Farook S A and Khan I A), pp. 457-463.
- Ashraf M A Kirti P B and Dineshkumar V 2007 Development of generic PTGS and PTGS constructs for silencing ricin and RCA genes in castor (*Ricinus communis* L.). In: *Extended Summaries – National Seminar on Changing global vegetable oils scenario: Issues and Challenges before India*, Directorate of Oilseeds Research, Hyderabad, p. 9.
- Ashri A and Knowles P F 1960 Cytogenetics of safflower (*Carthamus* L.) species and their hybrids. *Agronomy Journal* 52 : 11-17.
- Baker C M and Dyer W E 1996 Improvements in rooting regenerated safflower (*Carthamus tinctorius* L.) shoots. *Plant Cell Reports* 16 : 106-110.

- Balk S and Leaver C J 2001 The PET1-CMS mitochondrial mutation in sunflower is associated with premature programmed cell death and cytochrome c release. *Plant Cell* 13 : 1803-1818.
- Banga S and Raman R 1998 Biotechnology and Hybrid Breeding. In: Banga, S S. and Banga, S.K. (eds.) *Hybrid Cultivar Development*. Co-Pub. NPH, New Delhi and Springer Verlag, Germany, pp. 185-195.
- Banga S K Bhaskar P B and Banga S S 2006 Genetically engineered systems of male sterility. *Journal of Oilseeds Research* 23 : 1-7
- Baulcombe D 2005 RNA Silencing. *Trends in Biochemical Science* 30 : 290-293.
- Bayer M and Hess D 2005 Restoring full pollen fertility in transgenic male-sterile tobacco (*Nicotiana tabacum* L.) by Cre-mediated site-specific recombination. *Molecular Breeding* 15 : 193-203.
- Beals T P and Goldberg R B 1997 A novel cell ablation strategy blocks tobacco anther dehiscence. *Plant Cell* 9 : 1527-1545.
- Begu D Graves P V Domec C Arselin G Litvak S and Araya A 1990 RNA editing of wheat mitochondrial ATP synthase subunit 9: direct protein and cDNA sequencing. *Plant Cell* 2 : 1283-1290.
- Benne R Van der Burg J Brakenhoff J P Sloof P Van Boom J H and Tromp M C 1986 Major transcript of the frame-shifted COXII gene from trypanosome mitochondria contains four nucleotides that are not encoded in the DNA. *Cell* 46 : 819.
- Bhat S R and Srinivasan S 2002 Molecular and genetic analysis of transgenic plants: Consideration and approaches. *Plant Science* 163 : 673-681.
- Bisht N C Jagannath A Gupta V Burma P K and Pental D 2004 A two gene – two promoter system for enhanced expression of a restorer gene (Barstar) and development of improved fertility restorer lines for hybrid seed production in crop plants. *Molecular Breeding* 14 : 129–144.
- Bonhomme S Budar F Ferault M and Pelletier G 1991 A 2.5 kb *NcoI* fragment of Ogura radish mitochondrial-DNA is correlated with cytoplasmic male sterility in *Brassica* cybrids. *Current Genetics* 19 : 121–127.
- Bonnet E Van de Peer Y and Rouze P 2006 The small RNA world of plants. *New Phytologist* 171 : 451-468.

- Brummell D A Balint-Kurti P J Harpster M H Palys J M Oeller P W and Gutterson N 2003 Inverted repeat of a heterologous 3'-untranslated region for high-efficiency, high-throughput gene silencing. *Plant Journal* 33 : 793-800.
- Burgess D G Ralston E J Hanson W G Heckert M Jenq T Palys J M Tang K and Gutterson N 2002 A novel, two-component system for cell lethality and its use in engineering nuclear male-sterility in plants. *Plant Journal*. 31 : 113-125.
- Busi M V Zabaleta E J Araya A and Gomez-Casati D F 2006 Functional and molecular characterization of the frataxin homolog from *Arabidopsis thaliana*. *FEBS Letters* 576 : 141-144.
- Cao J S Yu X L Ye W Z Lu G and Xiang X 2006 Functional analysis of a novel male fertility *CYP86MF* gene in Chinese cabbage (*Brassica campestris* L. ssp. *chinensis* makino). *Plant Cell Reports* 24 : 715-723.
- Cacchetti V Pomponi M Altamura M M Pezzotti M Marsilio S D'Angeli S D Tornielli G B Constantino P and Cardarelli M 2004 Expression of *rolB* in tobacco flowers affects the coordinated processes of anther dehiscence and style elongation. *Plant Journal* 38 : 512-525.
- Chatterjee A K and Singh H P 1993 Plant regeneration from leaf calli of safflower. In: Li Dajue and Han Yuanzhou (eds), *Proceedings of Third International Safflower Conference Beijing, China (June 14-18, 1993)*. pp 139-143.
- Chase C D 2006 Genetically engineered cytoplasmic male sterility. *Trends in Plant Science* 11 : 7-9.
- Chavan V M 1961 Niger and Safflower. Indian Central Oilseeds Committee. Publication Hyderabad, India, pp 57-150.
- Cho H J Kim S Kim M and Kim B D 2001 Production of transgenic male sterile tobacco plants with the cDNA encoding a ribosome inactivating protein in *Dianthus sinensis* L. *Molecular Cell* 11 : 326-333.
- Cigan M A Unger-Wallace E and Haug-Collet K 2005 Transcriptional gene silencing as a tool for uncovering gene function in maize. *Plant Journal* 43 : 929-940.
- Coen E S and Meyerowitz E M 1991 The war of the whorls: Genetic interactions controlling flower development. *Nature* 353 : 31-37.
- Covello P S and Gray M W 1989 RNA editing in plant mitochondria. *Nature* 341 : 662-666.

- Deshmukh A K Patil R M and Nimbkar N 1989 Commercial scale exploitation of hybrid vigour in safflower using genetic male sterility system. In: Ranga Rao V and Ramachandram M (Eds.) 1991 Proc. Second International Safflower Conference, Hyderabad, India, pp. 163-167.
- Deshpande R B 1940. A sterile mutant in safflower (*Carthamus tinctorius* L.). Crop Science 9 : 370-371.
- De Block M Debrouwer D and Moens T 1994 The development of a nuclear male sterility system in wheat. Expression of the barnase gene under the control of tapetum specific promoters. Theoretical Applied Genetics 95 : 125-131.
- Dewey R E Levings C S and Timothy D H 1986 Novel recombinations in the maize mitochondrial genome produce a unique transcriptional unit in the Texas male-sterile cytoplasm. Cell 44 : 439-449.
- Doyle J J and Doyle J N 1987 A rapid DNA isolation protocol for small quantity of fresh leaf tissue. Phytochemistry Bulletin 19 : 11-15.
- Duroc Y Gaillard C Hiard S Tinchant C Berthome R Pelletier G and Budar F 2006 Nuclear expression of a cytoplasmic male sterility gene modifies mitochondrial morphology in yeast and plant cells. Plant Science 170 : 755-767.
- Fox T D and Leaver C J 1981 The *Zea mays* mitochondrial gene coding cytochrome oxidase subunit has an intervening sequence and does not contain TGA codons. Cell 26 : 315-323.
- George L and Rao P S 1982 *In vitro* multiplication of safflower (*Carthamus tinctorius* L.) through tissue culture. Proceedings of the Indian National Science Academy B (Biological Sciences) 48 : 791-794.
- Gray J Picton S Shabbeer J Schuch W and Grierson D 1992 Molecular biology of fruit ripening and its manipulation with antisense genes. Plant Molecular Biology 9 : 69-87.
- Grelon M Budar F Bonhomme S and Pelletier G 1994 Ogura cytoplasmic male-sterility (CMS)-associated *orf138* is translated into a mitochondrial membrane polypeptide in male-sterile *Brassica* cybrids. Molecular and General Genetics 243 : 540-547.
- Giege P and Brennicke A 1999 RNA editing in *Arabidopsis* effects 441 C to U changes in ORFs. Proceedings National Academy of Sciences USA 96 : 15324-15329.

- Goetz M Godt D E Guivarch A Kahmann U Chriqui D and Roitsch T 2001 Induction of male sterility in plants by metabolic engineering of the carbohydrate supply. Proceedings National Academy of Sciences USA 98 : 6522–6527.
- Goldberg R B Beals T P and Sanders P M 1993 Anther development: basic principles and practical applications. Plant Cell 5 : 1217-1229.
- Gomez - Casati D F Busi M V Schain N G Mouras A Zabaleta E J and Araya A 2002 A mitochondrial dysfunction induces the expression of nuclear encoded complex I genes in engineered male sterile *Arabidopsis thaliana*. FEBS letters 532 : 70-74.
- Gualberto J M Lamattina L Bonnard G Weil J H and Grienemberger J M 1989 RNA editing in wheat mitochondria results in the conservation of protein sequences. Nature 341 : 660-662.
- Gualberto J M Bonnard G Lamattina L and Grienemberger J M 1991 Expression of the wheat mitochondrial *nad3-rps12* transcription unit: Correlation between editing and mRNA maturation. Plant Cell 3 : 1109-1120.
- Guerineau F Sorensen A-M Fenby N and Scott R J 2003 Temperature sensitive diphtheria toxin confers conditional male-sterility in *Arabidopsis thaliana*. Plant Biotechnology Journal 1 : 33-42.
- Hanson M R Wilson R K Bentolila S Kohler R H and Chen H C 1999 Mitochondrial gene organization and expression in petunia male fertile and sterile plants. Journal of Heredity. 90: 362-368.
- Harinath D 2004 Cloning of *orfH522* from CMS sunflower (*Helianthus annuus* L.) for anther specific expression. M.Sc.(Ag.) Thesis, A.N.G.R.A.U., Hyderabad.
- Harrison H J and Harrison H Y 1970 Evaluation of pollen viability by enzymatically induced fluorescence; intracellular hydrolysis of fluorescein diacetate. Stain Technology 45 : 115-120.
- Hartl F U, Pfanner N, Nicholson D W and Neupert W 1989 Mitochondrial protein import. Biochemistry and Biophysics Acta 988 : 1-45.
- He S, Abad A R, Gelvin S B and Mackenzie S A 1996 A cytoplasmic male sterility-associated mitochondrial protein causes pollen disruption in transgenic tobacco. Proceedings of National Academy of Sciences, USA 93 : 11763-11768.

- Heaton T C and Knowles P F 1980 Registration of UC-148 and UC-149 male sterile safflower germplasm. *Crop Science* 20 : 554.
- Hegde D M 2006 Research achievements in safflower. All India Coordinated Research Project on Safflower, Directorate of Oilseeds Research, Hyderabad, India, p.111.
- Hiesel R Wissinger B Schuster W and Brennicke A 1989 RNA editing in plant mitochondria. *Science* 246 : 1632-1634.
- Heiser V Rasmusson A G Thieck O Brennicke A and Grohmann L 1997 Antisense repression of the mitochondrial NADH-binding subunit of complex I in transgenic potato plants affects male fertility. *Plant Science* 127 : 61-69.
- Hernould M Mouras A Litvak S and Araya A 1992 RNA editing of the mitochondrial *atp9* transcript from tobacco. *Nucleic Acids Research* 20 : 1809.
- Hernould M S Suharsono S Litvak A Araya A and Mouras 1993 Male-sterility induction in transgenic tobacco plants with an unedited *atp9* mitochondrial gene from wheat. *Proceedings National Academy Sciences USA* 90 : 2370-2374.
- Hernould M Zabaleta S E Carde J P Litvak S Araya A and Mouras A 1998 Impairment of tapetum and mitochondria in engineered male-sterile tobacco plants. *Plant Molecular Biology* 36 : 499-508.
- Hill A B 1989 Hybrid Safflower Breeding. In: *Proceedings of the Third International Safflower Conference*, Hyderabad, India, pp. 169-170.
- Hird D L Worrall D Hodge R Smartt S Paul W and Scott R 1993 The anther-specific protein encoded by the *Brassica napus* and *Arabidopsis thaliana* A6 gene displays similarity to b-1,3- glucanase expressed during tetrad dissolution. *Plant Journal* 4 : 1023-1033.
- Hird D L Paul W, Holloyoak J S and Scott 2000 The restoration of fertility in male sterile tobacco demonstrates that transgene silencing can be mediated by T-DNA that has no DNA homology to the silenced transgene. *Transgenic Research* 9 : 91-102.
- Horn R Höhler R H and Zetsche K 1991 A mitochondrial 16 kD protein is associated with cytoplasmic male sterility in sunflower. *Plant Molecular Biology* 17 : 29-36.
- Howad W and Kempken F 1997 Cell type-specific loss of *atp6* RNA editing in cytoplasmic male sterile *Sorghum bicolor*. *Proceedings National Academy Sciences USA* 94 : 11090-11095.

- Howad W and Tang H P Pring D R Kempken F 1998 Anther *atp6* RNA editing in cytoplasmic male sterile lines of *Sorghum bicolor*. In: Moller I M Gardestrom P Glimelius K and Glaser E (Eds.), Plant Mitochondria: From gene to function. Backhuis Publishers, Leiden, pp. 131-134.
- Irish V 1999 Petal and stamen development. Current Topics on Developmental Biology 41 : 133-161.
- Iwabuchi M Kyoizuka J and Shimamoto K 1993 Processing followed by complete editing of an altered mitochondrial *atp6* RNA restores fertility of cytoplasmic male sterile rice. Journal of European Molecular Biology Organization 12 : 1437-1446.
- Iwabuchi M Koizuka N Fujimoto H Sakai T and Imamura J 1999 Identification and expression of Kosenia radish (*Raphanus sativus*. L. cv. Kosenia) homologue of the ogura radish CMS associated gene, ORF138. Plant Molecular Biology 39 : 183-188.
- Jagannath, A., Arumugam, N., Gupta, V., Pradhan, A., Burma, P. K. and Pental, D. 2002. Development of transgenic barstar lines and identification of a male sterile barnase)/restorer (barstar) combination for heterosis breeding in Indian oilseed. Current Science 82 : 46-52.
- Jagannath A Bandyopadhyay P Arumugam N Gupta V Burma P K and Pental D 2001 The use of spacer DNA fragment insulates the tissue specific expression of a cytotoxic gene (barnase) and allows high-frequency generation of transgenic male sterile lines in *Brassica juncea* L. Molecular Breeding 8 : 11-23.
- Jambhale N D and Nerkar Y S 1983 Genetics of male sterility in safflower. Annual Report of Department of Plant Breeding, M.P.A.U., Parbhani.
- Johns C Lu M Lyznik A and Mackenzie S 1992 A mitochondrial DNA sequence is associated with abnormal pollen development in cytoplasmic male sterile pollen development in cytoplasmic male sterile bean plants. Plant Cell 4 : 435-449.
- Joshi B M Nerkar Y S and Jhambale N D 1983 Induced male sterility in safflower. Journal of Maharashtra Agricultural University 8 : 194-196.
- Kadam B S and Patnakar V K 1942 Natural cross-pollination in safflower. Indian Journal of Genetics and Plant Breeding 2: 69-76.
- Kalyani K Yamini K N and Dineshkumar V 2007 Study of expression pattern of TA29 promoter and coxIV presequence in safflower (*Carthamus tinctorius* L.). In:

- Extended Summaries – National Seminar on Changing global vegetable oils scenario: Issues and Challenges before India, Directorate of Oilseeds Research, Hyderabad, pp.12-14.
- Kaul M L H 1988 Male Sterility in Higher Plants, Berlin: Springer-Verlag p.1005.
- Keegstra K Olen L T and Theg S M 1989 Chloroplastic precursors and their transport across the envelop membrane. *Plant Molecular Biology* 40 : 471-501.
- Kempken F Howad W and Pring D R 1998 Mutations at specific *atp6* codons which cause human mitochondrial diseases also lead to male sterility in a plant. *FEBS Letters* 441 : 159-160.
- Khan M S 2005 Engineered male sterility. *Nature* 436 : 783-785.
- Kinney A J 1996 Development of genetically engineered soybean oils for food applications. *Journal of Food and Lipids* 3 : 273-292.
- Kirti P B Narasimhulu S B Prakash S and Chopra V L 1992 Somatic hybridization between *Brassica juncea* and *Moricandia arvensis* by protoplast fusion. *Plant Cell Reports* 11 : 318-321.
- Knutzen D S Thomson G A Radke S E Johnson W B Knauf V C and Kridl J C 1992 Modification of *Brassica* seed oil by antisense expression of a stearyl-acyl carrier protein desaturase. *Proceedings National Academy Sciences USA* 89 : 2624-2628.
- Koltunow M Jessie T Cox K H Wallroth M and Goldberg R B 1990 Different temporal and spatial gene expression pattern occur during anther development. *Plant Cell* 2 : 1201-1224.
- Kohler R H, Horn R, Lossl A and Zetsche K 1991 Cytoplasmic male sterility in sunflower is correlated with the co-transcription of a new open reading frame with the *atpA* gene. *Molecular and General Genetics* 66 : 369-36.
- Kriete G Niehaus K Perlick A M Puhler A and Broer I 1996 Male sterility in transgenic tobacco plants induced by tapetum specific deacetylation of the externally applied non-toxic compound N-acetyl-L-phosphinothricin. *Plant Journal* 9 : 809-818.
- Kugita M Yamamoto Y Fujikawa T Matsumoto T and Yoshinaga K 2003 RNA editing in hornwort chloroplasts makes more than half the genes functional. *Nucleic Acids Research* 31 : 2417-2423.

- Leshem B Shalev D P and Izhar S. 1988 Cytokinin as an inducer of vitrification in melon. *Annals of Botany* 61 : 255-260.
- L'Homme Y Stahl R J Li X Q Hameed A and Brown G G 1997. Brassica nap cytoplasmic male sterility is associated with expression of a mtDNA region containing a chimeric gene similar to the *pol* CMS-associated *orf224* gene. *Current Genetics* 31 : 325-335.
- Levings C S III 1993 Thoughts on cytoplasmic male sterility in cms-T maize. *Plant Cell* 5 : 1285-1290.
- Luo H Lyznik L A Gidoni D and Hodges T K 2000 FLP-mediated recombination for use in hybrid plant production. *Plant Journal* 23 : 423-430.
- Lu B and Hanson M R 1992 A single nuclear gene specifies the abundance and extent of RNA editing of a plant mitochondrial transcript. *Nucleic Acids Research* 20 : 5699-5703.
- Lu B and Hanson M R 1996 Fully edited and partially edited *nad9* transcripts differ in size and both are associated with polysomes in potato mitochondria. *Nucleic Acids Research* 24 : 1369-1374.
- Maarse A C Van Loon A P Riezman H Gregor I Schatz G and Grivell L A 1984 Subunit IV of yeast cytochrome C oxidase: cloning and nucleotide sequencing of the gene and partial amino acid sequencing of the mature protein. *Journal of European Molecular Biology Organization* 3 : 2831-2837.
- Mackenzie S and Chase C 1990 Fertility restoration is associated with loss of a portion of the mitochondrial genome in cytoplasmic male-sterile common bean. *Plant Cell* 2 : 905-912.
- Maier R M Neckermann K Igloi G L and Kossel H 1995 Complete sequence of the maize chloroplast genome: gene content, hotspots of divergence and fine tuning of genetic information by transcript editing. *Journal of Molecular Biology* 251 : 614-628.
- Mandal A K A Chatterjee A K and Gupta S D 1995 Direct somatic embryogenesis and plantlet regeneration from cotyledonary leaves of safflower. *Plant Cell, Tissue and Organ Culture* 43 : 287-289.
- Mandal A K A and Guptha S D 2001 Direct shoot organogenesis and plant regeneration in safflower. *In vitro Cellular and Developmental Biology – Plant* 37 : 50-54.

- Mandal A K A and Guptha S D 2002 Direct somatic embryogenesis of safflower - a scanning electron microscopic study. *Current Science* 83 : 1138-1140.
- Mansoor S Amin I Hussain M Zafar Y and Briddon R W 2006 Engineering novel traits in plants through RNA interference. *Trends in Plant Science* 11 : 559-565.
- Mariani C De Beuckeleer M Truettner J Leemans J and Goldberg R B 1990 Induction of male sterility in plants by a chimaeric ribonuclease gene. *Nature* 347 : 737-741.
- Mariani C Gossele V Beuckeler M D Block M D Goldberg R B Greff W D and Leemans J 1992 A chimeric ribonuclease-inhibitor gene restores fertility to male sterile plants. *Science* 357 : 384-387.
- Mass S and Rich A 2000 Changing genetic information through RNA editing. *Bio Essays* 22 : 790-802.
- Moneger F Smart J C and Leaver C J 1994 Nuclear restoration of cytoplasmic male sterility in sunflower is associated with the tissue specific regulation of a novel mitochondrial gene. *Journal of European Molecular Biology Organization* 13 : 8-17.
- Mower J P 2005 PREP-Mt: predictive RNA editor for plant mitochondrial genes. *BMC Bioinformatics* 6 : 96
- Murashige T and Skoog F 1962 A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum* 15 : 473-497.
- Matern U and Kneusel R E 1993 The use of recombinant DNA techniques to confer resistance to the *Alternaria* leaf spot disease of safflower. In: Dajue L, Yunzhou (Eds.), *Proceedings of the 3rd International Safflower Conference*, Beijing, China, pp. 807-815.
- Nikam T D and Shitole M G 1999 In vitro culture of Safflower *L. cv. Bhima*: initiation, growth optimization and organogenesis. *Plant Cell, Tissue and Organ Culture* 55 : 15-22.
- Notsu Y Masood S Nishikawa T Kubo N Akiduki G Nakazono M Hirai A and Kadowaki K 2002 The complete sequence of the rice (*Oryza sativa* L.) mitochondrial genome: frequent DNA sequence acquisition and loss during the evolution of flowering plants. *Molecular Genetics and Genomics* 268 : 434-445.
- Orlikowska T K and Dyer W E 1993 *In vitro* regeneration and multiplication of safflower (*Carthamus tinctorius* L.). *Plant Science* 93 : 151-157.

- Orlikowska T K Cranston J H and Dyer W E 1995 Factors influencing *Agrobacterium tumefaciens* mediated transformation of the Safflower cv 'Centennial'. Plant Cell, Tissue and Organ Culture 40 : 85-91.
- Ow D W and Medburry S L 1995 Genome manipulation through site-specific recombination. Critical Review on Plant Sciences 14 : 239-261.
- Pandey L B Zehr B E Reddy K V S Babu S S and Raunt V K 2007 MRSA-521- First commercial CMS based hybrid in safflower. Extended Summaries - National Seminar on "Changing global vegetable oils scenario: Issues and Challenges before India", Directorate of Oilseeds Research, Hyderabad, pp. 142-143.
- Paques M and Boxus P 1987 A model to learn 'vitrification', the rootstock apple M.26. Present Results. Acta Horticultura 212 : 193-210.
- Pesce A S and Grabau E A 1993 RNA editing of the soybean mitochondrial *atp9* transcript. Plant Physiology. 103 : 1457-1458.
- Perrotta G Malek O Hieser V Brennicke A Grohmann L and Quagliariello C 1996 RNA editing in the *cox3* mRNA of *Magnolia* is more extensive than in other dicot or monocot plants. Biochimica et Biophysica Acta 1307 : 254-258.
- Prasad B R Khadeer M A Seeta P and Anwar S Y 1991 *In vitro* induction of androgenic haploids in safflower (*Carthamus tinctorius* L.). Plant Cell Reports 10 : 48-51.
- Radhika K Sujatha M and Nageshwar Rao T 2006 Thidiazuron stimulates adventitious shoot regeneration in different safflower explants. Biologia Plantarum 50 : 174-179.
- Ramachandram M and Sujatha M 1991 Development of genetic male sterile lines in safflower. Indian Journal of Genetics and Plant Breeding 51 : 268-269.
- Rani K J and Rao T N 1998 Callus differentiation in tissue cultures of safflower. Journal of Oilseed Research 15 : 258-260.
- Rani K J, Rao T N Raghunatham G and Rao P V 1996 Studies on callus growth and differentiation in safflower. Indian Journal of Genetics 56 : 458-461.
- Rankin C T Cutright M T Makaroff C A 2001 Characterization of the radish mitochondrial *nad3/rps12* locus: analysis of recombination repeats and RNA editing. Current Genetics 29 : 564-571
- Rao S K and Rohini V K 1999 Gene transfer in to Indian cultivars of safflower (*Carthamus tinctorius* L.) using *Agrobacterium tumefaciens*. Plant Biotechnology 16 : 201-206.

- Rao N N Sujatha M and Kumar V D 2007 Establishment of regeneration and transformation protocols in safflower (*Carthamus tinctorius* L.). Extended Summaries - National Seminar on "Changing global vegetable oils scenario: Issues and Challenges before India", Directorate of Oilseeds Research, Hyderabad, pp. 14-16.
- Reddy 1997 Restoration of fertility by antisense RNA in genetically engineered male sterile tobacco plants. *Molecular and General Genetics* 237 : 385-394.
- Richharia 1941. Progress report of oilseed research scheme. Central Province, 1941. W.C.P. Plant Breeding and Genetics in India. Scientific Book Co., Patna. pp. 130-133.
- Rohini V K and Rao K S 2000 Embryo Transformation, A practical approach for realizing transgenic plants of safflower (*Carthamus tinctorius* L.). *Annals of Botany* 86 : 1043-1049.
- Roque E Gomez M D Ellul P Madueno F W Beltran J and Canas L A 2007 The PsEND1 promoter a novel tool to produce genetically engineered male sterile plants by early anther ablation. *Plant Cell Reports* 26 : 313-325.
- Ruiz O N and Daniell H 2005 Engineering Cytoplasmic Male Sterility via the Chloroplast Genome by Expression of β -Ketothiolase. *Plant Physiology* 138 : 1232-1246.
- Rurek M Szklarczyk M Adamczyk N Michalik B and Augustyniak H 2001 Differences in editing of mitochondrial *nad3* transcripts from CMS and fertile carrots. *Acta Biochimica Polonica* 48 : 711-717.
- Sabar M Gagliardi D Balk J and Leaver C J 2003 ORFB is a subunit of F(1)F(O)- ATP synthase: insight into the basis of cytoplasmic male sterility in sunflower. *European Molecular Biology Organization Reports* 4 : 1-6.
- Salazar R A, Pring D R and Kempken F 1991 Editing of mitochondrial *atp9* transcripts from two sorghum lines. *Current Genetics* 20 : 483-486.
- Sambrook J and Russel DW 2001 *Molecular Cloning : A laboratory Manual*, 3rd edition. Volume I-III. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
- Sanders P M Bui Q A Le B H and Goldberg R B 2005 Differentiation and degeneration of cells that play a major role in tobacco anther dehiscence. *Sexual Plant Reproduction* 17 : 219-241.
- Sawhney V K and Shukla A 1994 Male sterility in flowering plants: Are plant growth substances involved? *Americal Journal of Botany* 81 : 1640-1647.

- Schmitz U K and Lonsdale D M 1989 A Yeast Mitochondrial Presequence Functions as a signal for Targeting to Plant Mitochondria *in vivo*. Plant Cell 1 : 783-791.
- Schmulling T Schell J and Spena A 1988 Single genes from *Agrobacterium rhizogenes* influence plant development. Journal of European Molecular Biology Organization 7 : 2621-2629.
- Schmülling T Röhrig H Pilz S Walden R and Schell J 1993 Restoration of fertility by antisense RNA in genetically engineered male sterile tobacco plants. Molecular and General Genetics 237 : 385-394.
- Scott R Hodge R Paul W and Draper J 1991 The molecular biology of anther differentiation. Plant Science 80 : 167-191.
- Scott R J Spielman M and Dickinson H J 2004 Stamen structure and function. Plant Cell 16: S46-S60.
- Shivanna K R 2002 Pollen Biology and Biotechnology Oxford and IBH Publishing Co. Pvt. Ltd.
- Skoog F and Miller C O 1957 Chemical regulation of growth and organ formation in plant tissues cultured *in vitro*. In: Biological action of growth substances. Symposia Society for Experimental Biology 11 : 118-131.
- Schuster W Unseld M Wissinger B and Brennicke A 1990 Ribosomal protein S14 transcripts are edited in *Oenothera* mitochondria. Nucleic Acids Research 18 : 229-233.
- Schuster W and Brennicke A 1990 RNA editing of ATPase subunit 9 transcripts in *Oenothera* mitochondria. FEBS Letters 268 : 252-256.
- Singh H P 1991 Morphogenetic potential of callus and organ cultures of safflower. Narendra Deva Journal of Agricultural Research 6 : 163-167.
- Singh M and Brown G G 1991 Suppression of cytoplasmic male sterility by nuclear genes alters expression of a novel mitochondrial gene region. Plant cell 6 : 811-825.
- Singh V 1996 Inheritance of genetic male sterility in safflower. Indian Journal of Genetics and Plant Breeding 56 : 454-458.
- Singh V 1997 Identification of genetic linkage between male sterility and dwarfness in safflower. Indian Journal of Genetics and Plant Breeding 57 : 327-332.

- Singh V Deshpande M B Galande M K Deshmukh S R and Nimbkar N 2000 Current status of research and development in safflower hybrid in India. Extended summaries. National Seminar on "Oilseeds and Oils - Research and Development Needs in the Millennium". February 2-4, 2000. Indian Society of Oilseeds Research, DOR, Hyderabad, p. 62.
- Smith C Watson C Bird C Ray J W S and Grierson D 1990 Expression of a truncated tomato polygalacturonase gene inhibits expression of the endogenous gene in transgenic plants. *Molecular and General Genetics* 224 : 477-481.
- Sollner T Griffiths G Pfaller R Pfanner N and Neupert W 1989 Mom19, an import receptor for mitochondrial precursor proteins. *Cell* 59 : 1061-1070.
- Spena A Estruch J J Prensen E Nacken W Van Onckelen H and Sommer H 1992 Anther specific expression of the rol B gene of *Agrobacterium rhizogenes* increase IAA content in anthers and alters anther development in whole flower growth. *Theoretical and Applied Genetics* 84 : 520-527.
- Spena A Schmulling T Konec C and Shell J 1987 Independent and synergistic activity of rol A, rol B and rol C loci in stimulating abnormal growth of plants. *The Journal of European Molecular Biology Organization* 6 : 3891-3899.
- Stahl R Sun S Homme L Y Ketela T and Brown G G 1994 RNA editing of transcripts of a chimeric mitochondrial gene associated with cytoplasmic male-sterility in *Brassica*. *Nucleic Acids Research* 22 : 2109-2113.
- Sugaira M 1995 The chloroplast genome. *Essays Biochemistry* 30 : 49-57.
- Sujatha M 2007 Advances in safflower biotechnology. In: *Functional Plant Science and Biotechnology* 1 p. 160-170.
- Sujatha M and Suganya A 1996 *In vitro* organogenic comparison of different seedling tissues of safflower (*Carthamus tinctorius* L.). *Sesame and Safflower Newsletter* 11: 85-90.
- Suzuki T Kazama S Hirai A Akihama T and Kadowaki K 1991 The rice mitochondrial *nad3* gene has an extended reading frame at its 5' end: Nucleotide sequence analysis of rice *trnS*, *nad3* and *rps12*. *Current Genetics* 20 : 331- 337.
- Tang H V Pring D R Muza F R and Yan B 1996 Sorghum mitochondrial *orf25* and a related chimeric configuration of a male sterile cytoplasm. *Current Genetics* 29: 265-274.

- Tejovathi G and Anwar S Y 1984 *In vitro* induction of capitula from cotyledons of *Carthamus tinctorius* (safflower). Plant Science Letters 36 : 165-168.
- Tejovathi G and Anwar S Y 1987 Plant regeneration from cotyledonary culture of safflower. In: Proceedings of National Symposium "Plant Cell Tissue Culture of Economically Important Plants", G.M.Reddy (Ed.) ,Hyderabad, India, pp. 347-354.
- Tejovathi G and Anwar S Y 1993 Anatomical studies on *in vitro* induced morphological variants showing shoot bud development in safflower (*Carthamus tinctorius* L.). Advances in Plant Sciences 6 : 293-298.
- Thorsness M K Kandasamy M K Nasrallah M E Nasrallah J B 1993 A *Brassica* S-locus gene promoter targets toxic gene expression and cell death to the pistil and pollen of transgenic *Nicotiana*. Developmental Biology 143 :173-184.
- Touzet P and Budar F 2004 Unveiling the molecular arms race between two conflicting genomes in cytoplasmic male sterility. Trends in Plant Science 19 : 568-570.
- van der Meer I M Stam M E van Tunen A J Mol J N M and Stuitje A R 1992 Antisense inhibition of flavonoid biosynthesis in petunia anthers results in male sterility. Plant Cell 4 : 253-262.
- Veluthambi K, Gupta A K and Sharma A 2003 The current status of plant transformation technologies. Current Science 84 : 368-380.
- Vijayakumar J and Kumari B D R 2005 Effect of phytohormones on multiple shoot bud induction in cv. NARI-6 of safflower (*Carthamus tinctorius* L.). Journal of Plant Biotechnology 7 : 1-5.
- Wang, Z., Zou, Y., Li, X., Zhang, Q., Chen, L., Wu, H., Su, D., Chen, Y., Guo, J., Luo, D., Long, Y., Zhong, Y. and Liu, Y. 2006. Cytoplasmic male sterility of Rice with Boro II cytoplasm is caused by a cytotoxic peptide and is restored by two related PPR motif genes via distinct modes of mRNA silencing. Plant Cell, 18 : 676-687.
- Waterhouse P M and Helliwell C A 2002 Exploring plant genomes by RNA-induced gene silencing. Nature Reviews of Genetics 4 : 29-38.
- Wintz H and Hanson M R 1991 A termination codon is created by RNA editing in the petunia mitochondrial *atp9* gene transcript. Current Genetics 19 : 61-64.

- Wissinger B Schuster W and Brennicke A 1990 Species-specific RNA editing patterns in the mitochondrial *rps13* transcripts of *Oenothera* and *Daucus*. *Molecular and General Genetics* 224 : 386–395.
- Worrall D Hird D L Hodge R Paul W Draper J and Scott R 1992 Premature dissolution of the microsporocyte callose wall causes male sterility in transgenic tobacco. *Plant Cell* 4 : 759-771.
- Xu H Knox RB Taylor PE and Singh MB 1995 *Bcp1*, a gene required for male fertility in *Arabidopsis*. *Proceedings National Academy of Sciences USA* 92: 2106-2110.
- Young E G and Hanson M R 1987 A fused mitochondrial gene associated with cytoplasmic male sterility is developmentally regulated. *Cell* 50: 41-49.
- Ying M Dyer W T and Bergman J 1992 *Agrobacterium tumefaciens* mediated transformation of Safflower (*Carthamus tinctorius* L.) cv. 'Centennial'. *Plant Cell Reports* 11 : 581-585.
- Yui R Iketani S Mikami T and Kubo T 2003 Antisense inhibition of mitochondrial pyruvate dehydrogenase E1 α subunit in anther tapetum causes male sterility. *Plant Journal* 34 : 57-66.
- Zabaleta E Mouras A Hernould M Suharsono S and Araya A 1996 Transgenic male-sterile plant induced by an unedited *atp9* gene is restored to fertility by inhibiting its expression with antisense RNA. *Proceedings National Academy of Sciences USA* 93 : 11259–11263.
- Zhan X Wu H and Chung AY 1996 Nuclear male sterility induced by pollen specific expression of a ribonuclease. *Sexual Plant Reproduction* 9 : 35-43.
- Zhanming H and Biwen H 1993 The tissue culture of safflower and its histological and cytological study. In: Dajue L, Yunzhou H (Eds.) *Proceedings of the 3rd International Safflower Conference*, Beijing, China, pp. 184-194.
- Zhang Y Shewry P R Jones H Barcelo P Lazzeri P A and Halford N G 2001 Expression of antisense SnRK1 protein kinase sequence causes abnormal pollen development and male sterility in transgenic barley. *The Plant Journal* 28 : 431-441.
- Wilson, Z. A. and Yang, C. 2004. Plant gametogenesis: conservation and contrasts in development. *Reproduction* 128 : 483–492.

Zubko M K 2004 Mitochondrial tuning fork in nuclear homeotic functions. Trends in Plant Science 9 : 61-64.

www.ncbi.nlm.nih.gov

<http://regional.org.au/au/gcirc/4/493.htm>

http://www.unizh.ch/botinst/Molec_Website/bkeller_home.html

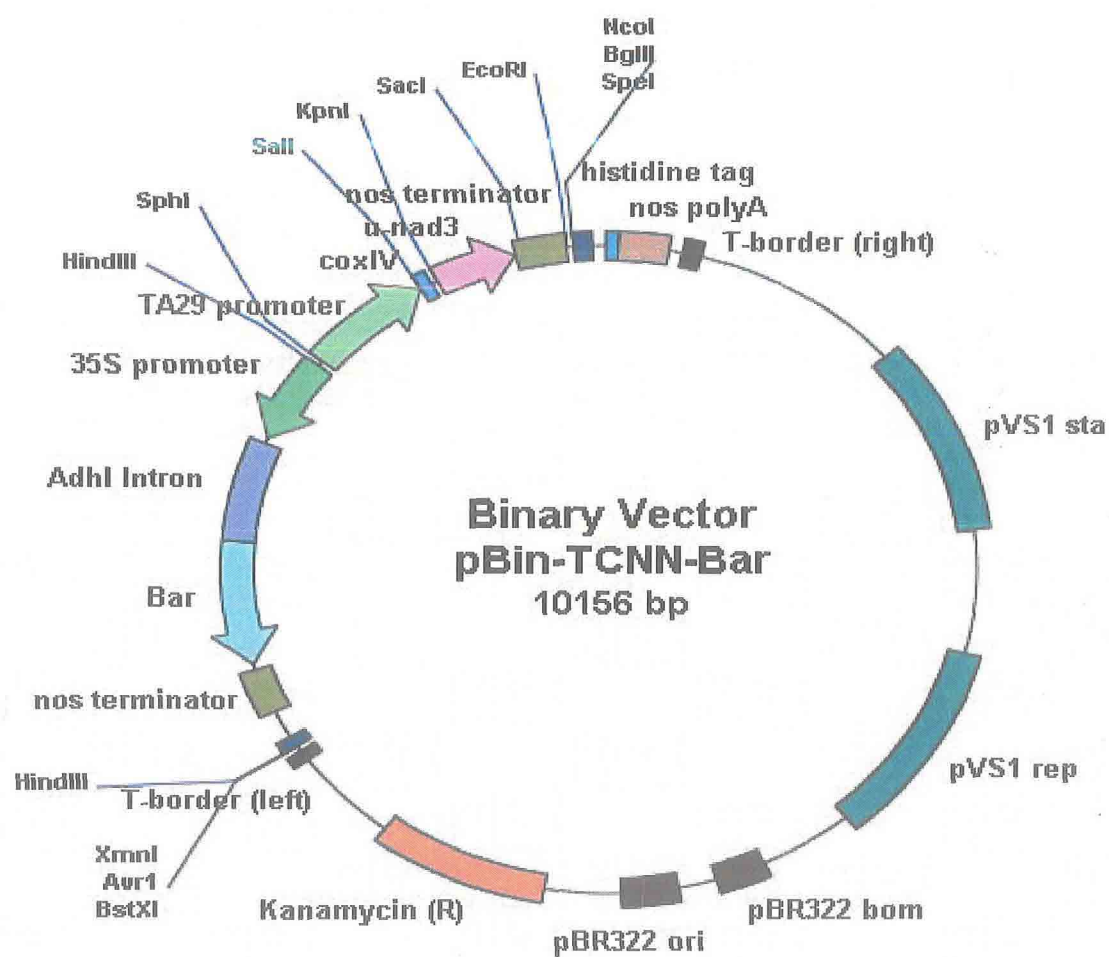
<http://www.regional.org.au/au/gcirc/4/245.htm#TopOfPage>

http://www.avesthagen.com/docs/newsletters/jan_feb_2005.pdf

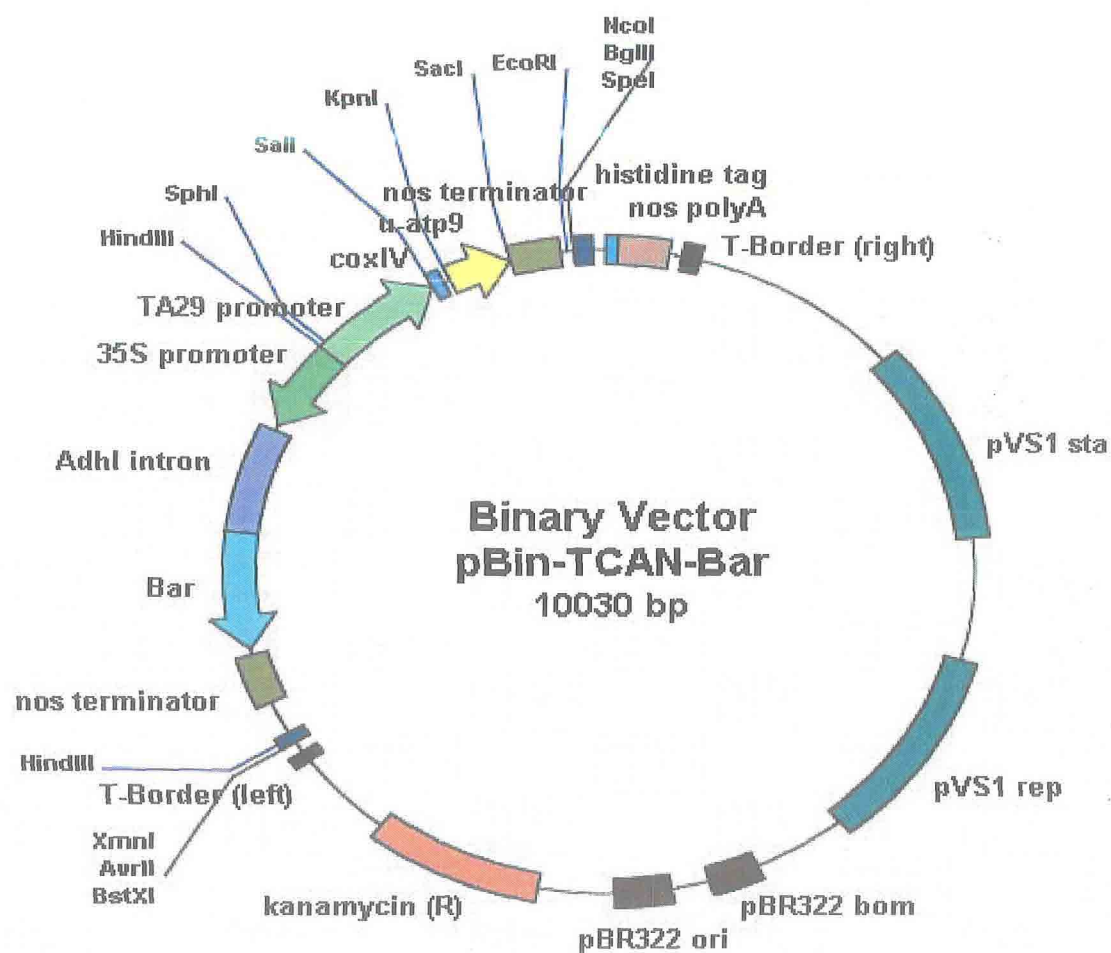
The pattern of "Literature cited" presented above is in accordance with the "Guidelines" for thesis presentation for Acharya N. G. Ranga Agricultural University, Hyderabad.

APPENDICES

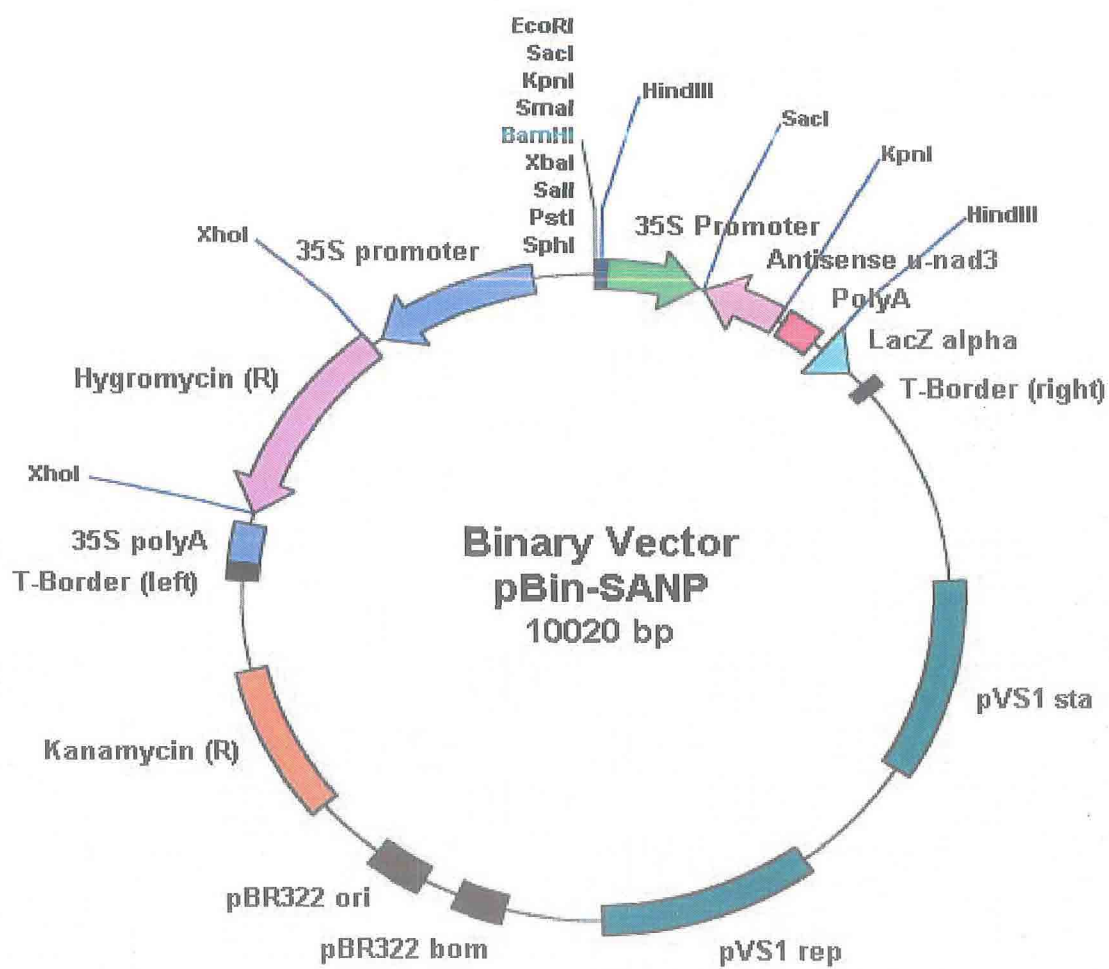
Appendix 1 Plasmid map of pBin-TCNN-Bar



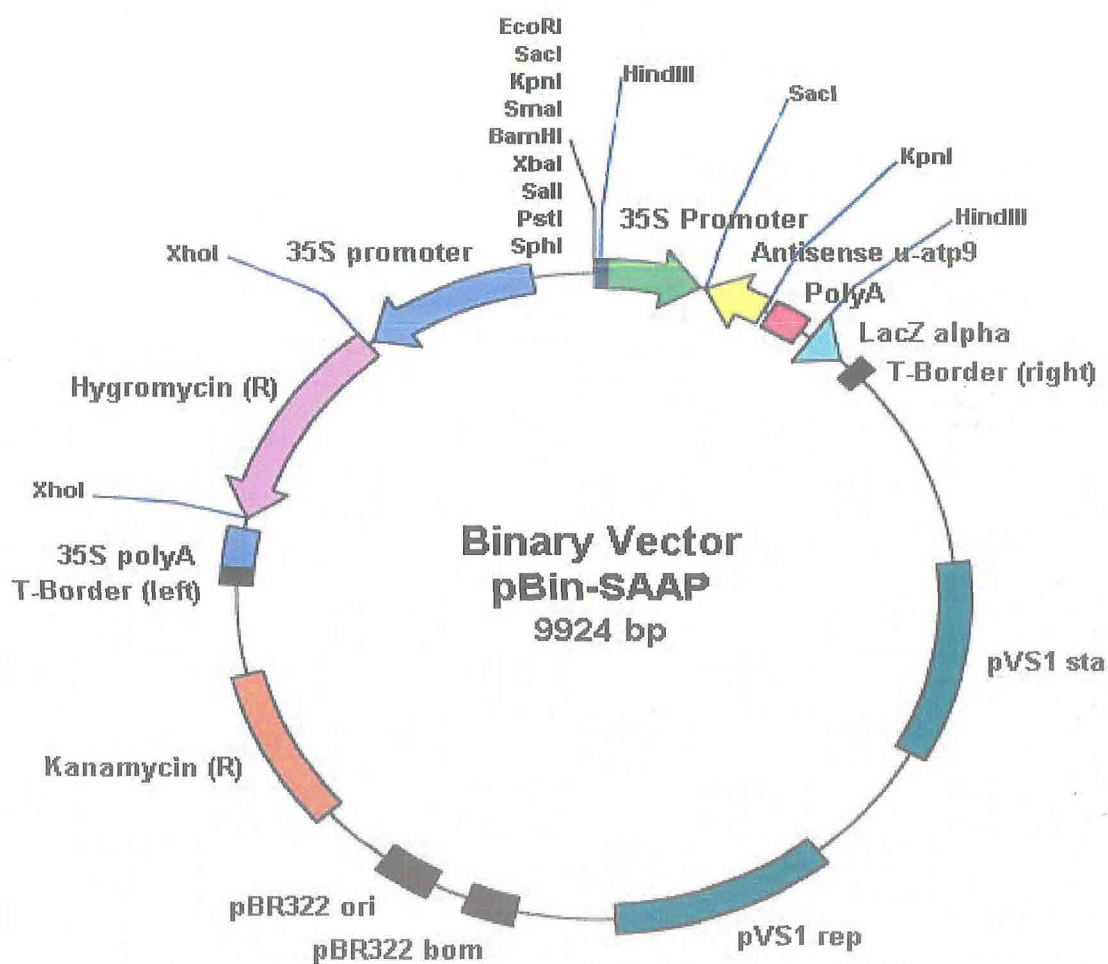
Appendix II Plasmid map of pBin-TCAN-Bar



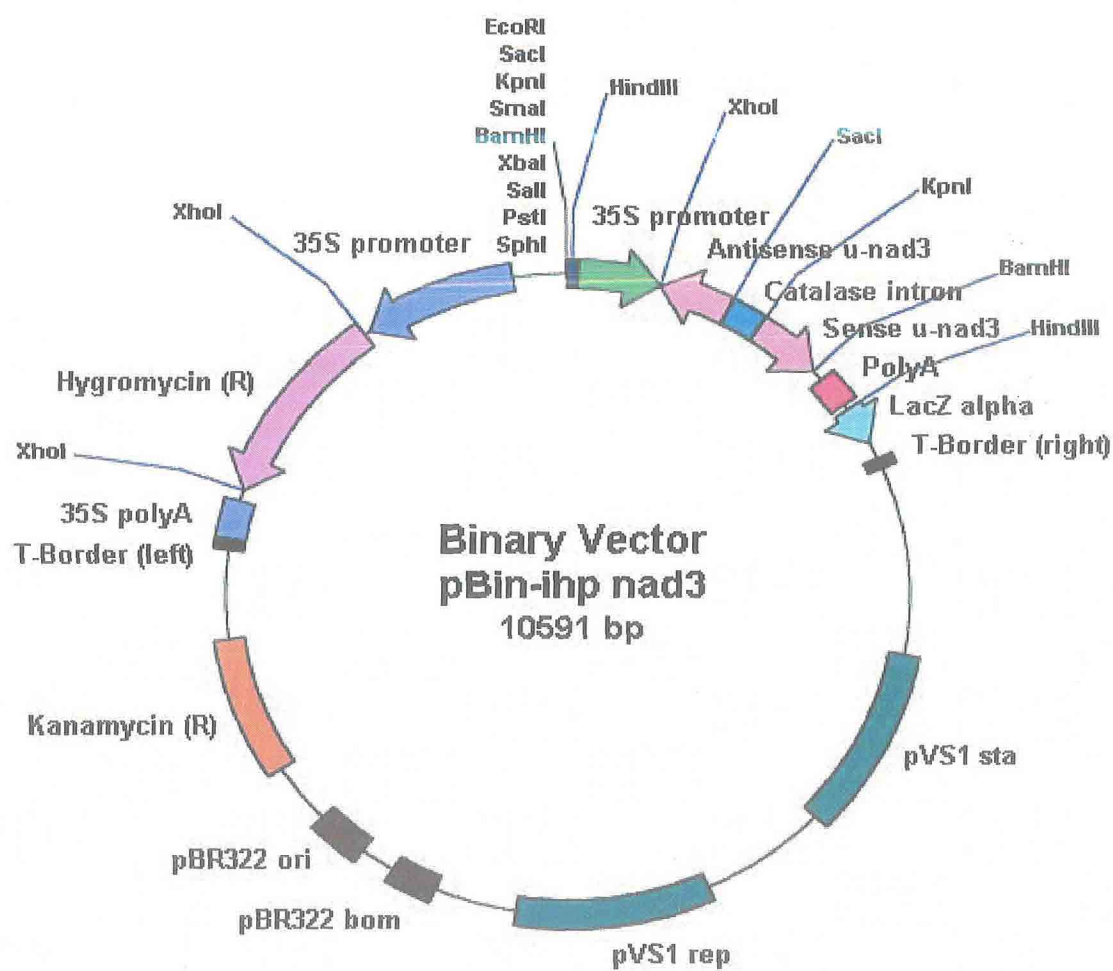
Appendix III Plasmid map of pBin-SANP



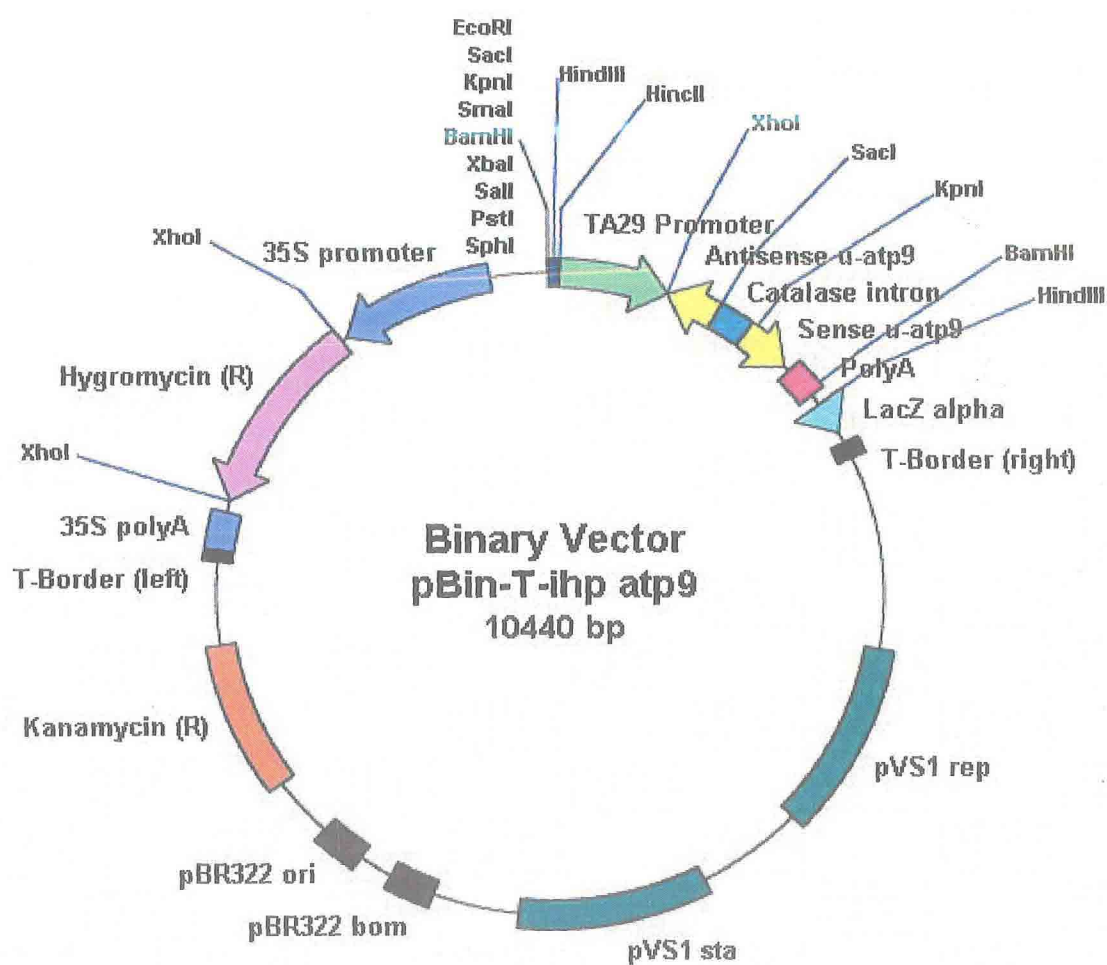
Appendix IV Plasmid map of pBin-SAAP



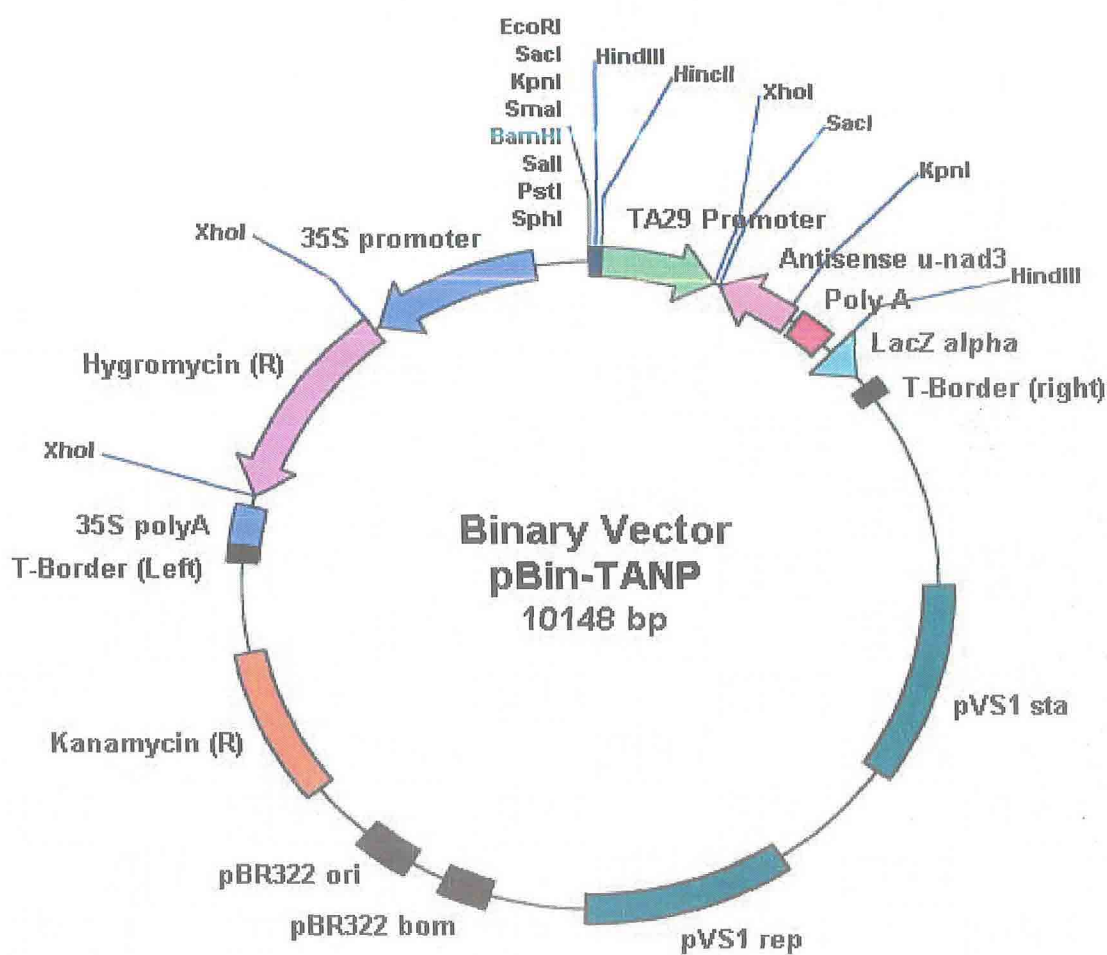
Appendix V Plasmid map of pBin-ihp *nad3*



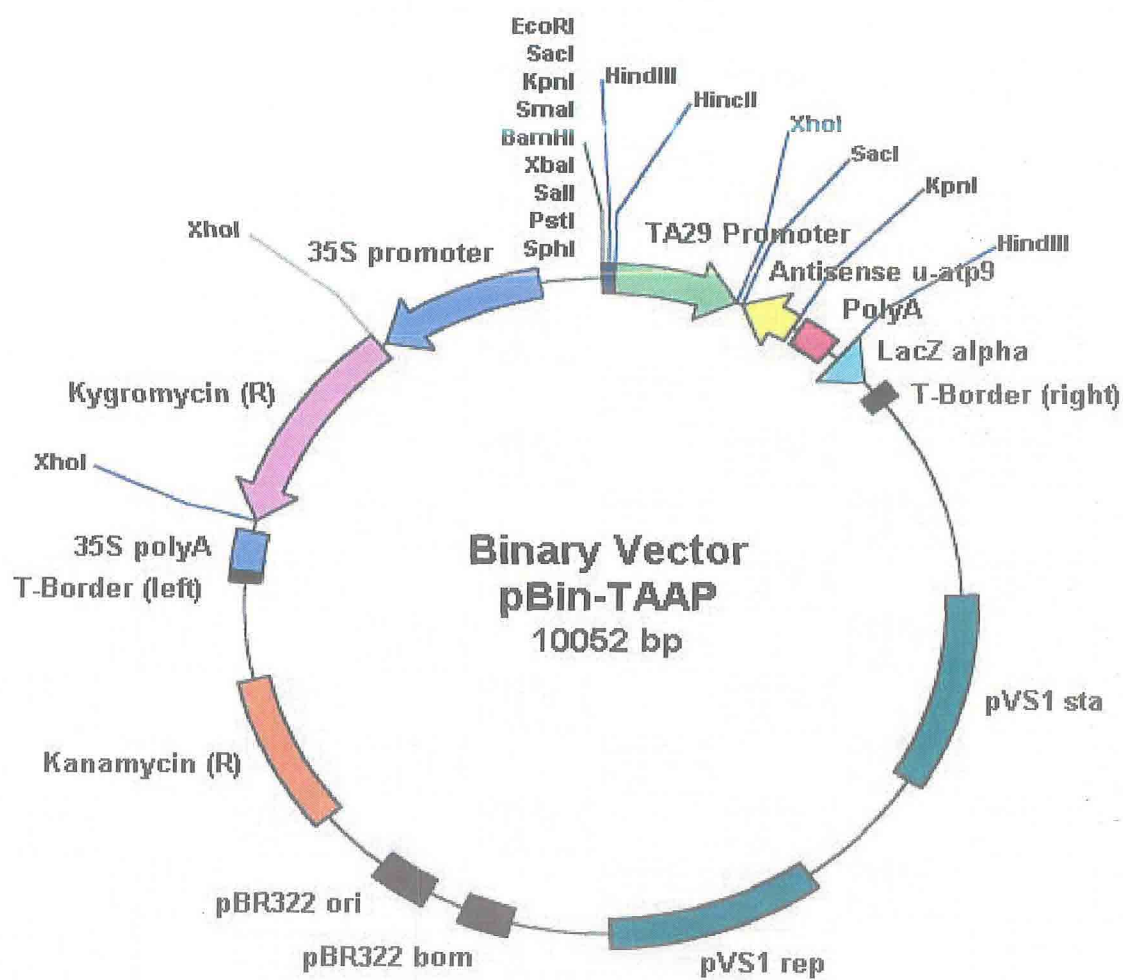
Appendix VI Plasmid map of pBin-ihp *atp9*



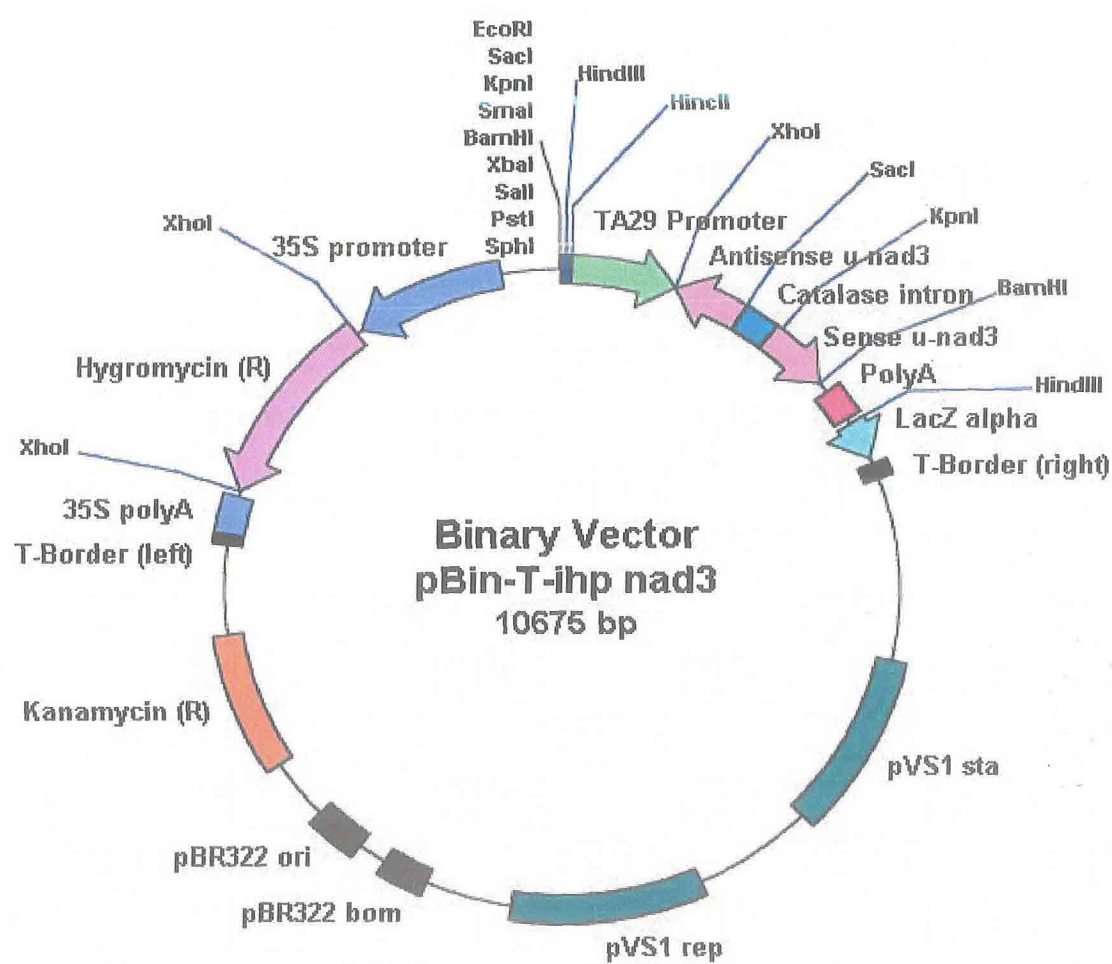
Appendix VII Plasmid map of pBin-TANP



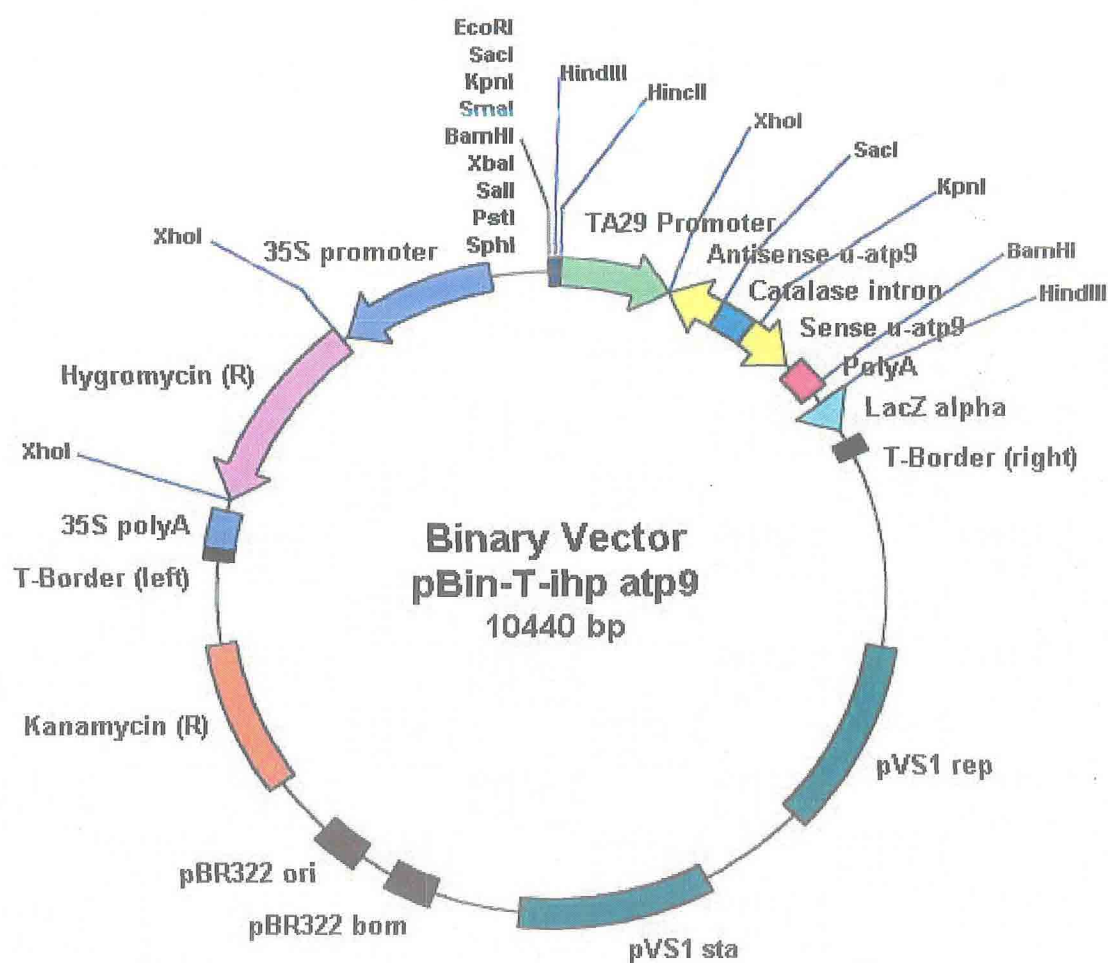
Appendix VIII Plasmid map of pBin-TAAP



Appendix IX Plasmid map of pBin-T-ihp *nad3*



Appendix X Plasmid map of pBin-T-ihp *atp9*



ANGRAU Central Library
HYDERABAD-500 030.
ACC. No. D-8088
Date. 9.1.08