

# TABLE OF CONTENTS

<b>1. INTRODUCTION</b>	1
<b>2. REVIEW OF LITERATURE</b>	13
<b>2.1. <i>Agrobacterium</i>-mediated transformation in cereals</b>	13
<b>2.2. Tissue culture and <i>in vitro</i> regeneration studies</b>	22
2.2.1. Role of genotype on plant regeneration	22
2.2.2. Role of growth regulators in <i>in vitro</i> plant regeneration	22
2.2.3. Genetic variability in plants regenerated <i>in vitro</i>	24
<b>2.3. Successful transformation strategies</b>	25
2.3.1. Choice of the explants used for transformation and plant regeneration	26
2.3.2. Choice of promoters	27
2.3.3. Choice of reporter gene	31
2.3.4. Choice of selectable marker for transformation	33
2.3.4. Selection of transgenics	35
<b>2.4. Analysis of transgenic plants</b>	36
2.4.1. Detection of transgene in the transformants	36
2.4.2. Transgene inheritance studies	37
2.4.3. Mechanisms of transgene integration into a host genome	37
2.4.4. Genetic instability in transformants	38
2.4.5. Transgene silencing	39
<b>2.5. <i>Bacillus thuringiensis</i>—a gain to genetic transformation</b>	41
2.5.1. Classification of <i>cry</i> genes	42
2.5.2. Mode of action of Bt protein on ingested larva	45
2.5.2.1. P <sup>H</sup> of the mid gut	45
2.5.2.2. Mid-gut enzymes	46
2.5.2.3. Mid-gut structure	46
2.5.3. Genes from <i>Bacillus thuringiensis</i>	47
2.5.4. <i>Bt</i> genes for resistance against stem borers	49
2.5.5. Transgenic plants with synthetic <i>Bt</i> genes	50
<b>2.6. Genetic transformation of sorghum</b>	51

<b>3. MATERIALS AND METHODS</b>	62
<b>3.1. Experimental material</b>	62
3.1.1. Plant material	62
3.1.2. Glassware	62
3.1.3. Chemicals	62
<b>3.2. Culture medium and conditions</b>	63
3.2.1. Sterilization	63
3.2.2. Aseptic conditions	63
3.2.3. Maintenance of cultures	63
<b>3.3. <i>In vitro</i> regeneration system for sorghum</b>	64
3.3.1. Preparation of shoot apical meristem	64
3.3.2. Initiation of shoot buds	64
3.3.3. Plant regeneration <i>in vitro</i> via somatic embryogenesis	64
3.3.4. Root induction and hardening of the plantlets	65
<b>3.4. <i>Agrobacterium</i> vectors and strains</b>	65
3.4.1. Plasmid vectors	65
3.4.1.1. pCAMBIA3300:Ubi:cry1Aa	65
3.4.1.2. pCAMBIA1301	68
3.4.2. Bacterial stains	68
3.4.2.1. DH5 $\alpha$	68
3.4.2.2. EHA105	68
<b>3.5. Transformation of competent <i>E. coli</i></b>	68
3.5.1. Preparation of <i>E. coli</i> competent cells	68
3.5.2. Transformation of <i>E. coli</i>	69
<b>3.6. Transformation of competent <i>Agrobacterium</i></b>	69
<b>3.7 Isolation and purification of plasmid DNA</b>	70
<b>3.8. <i>Agrobacterium</i>- mediated transformation</b>	74
3.8.1. Sensitivity of shoot apices to phosphinothricin	74
3.8.2. Optimization of condition for infection and co-cultivation	74
3.8.2.1. <i>Agrobacterium</i> infection	74
3.8.2.2. Co-cultivation	75
3.8.3. Elimination of <i>Agrobacterium</i> using antibiotics	75
<b>3.9. Histochemical <i>gus</i> assay</b>	75
<b>3.10. <i>Agrobacterium</i>-mediated transformation using <i>cry1Aa</i> gene construct</b>	76

3.10.1. <i>Agrobacterium</i> infection and co-cultivation	76
3.10.2. Selection and regeneration of putative transgenics	76
3.10.3. Phosphinothricin selection strategy	77
3.10.4. Root induction and acclimatization	77
<b>3.11. Isolation and purification of sorghum genomic DNA</b>	<b>77</b>
3.11.1. Isolation of sorghum genomic DNA	77
3.11.2. Purification of sorghum genomic DNA	78
3.11.3. Qualitative analysis of sorghum genomic DNA	79
3.11.4. Quantitative analysis of sorghum genomic DNA	79
<b>3.12 Molecular analysis of transgenic plants</b>	<b>80</b>
3.12.1. Polymerase chain reaction (PCR)	80
3.12.2. Southern blot analysis	81
3.12.2.1. Restriction digestion of plant genomic DNA	82
3.12.2.2. Precipitation of digested DNA	82
3.12.2.3. Transfer of digested DNA to nylon membrane	83
3.12.2.4. Preparation of purified probe for hybridization	84
3.12.2.5. Radioisotope labelling of PCR probe	85
3.12.2.6. Pre-hybridization	85
3.12.2.7. Hybridization	85
3.12.2.8. Post-hybridization	85
3.12.2.9. Autoradiography	86
3.12.2.10. Development of x-ray film	86
<b>3.13. Inheritance of <i>cryIAa</i> gene in T<sub>1</sub> generation</b>	<b>86</b>
<b>3.14. RT-PCR analysis</b>	<b>86</b>
3.14.1. Extraction and purification of sorghum RNA	86
3.14.2. Quantitative analysis of sorghum RNA	88
3.14.3. RT-PCR	88
<b>3.15. Insect bioassays</b>	<b>89</b>
3.15.1. Insect diet preparation	89
3.15.2. Leaf disc bioassay	90
3.15.3. Whole plant bioassay	91
<b>3.16. Statistical analysis</b>	<b>92</b>

<b>4. RESULTS</b>	93
<b>4.1. <i>In vitro</i> regeneration via somatic embryogenesis</b>	93
4.1.1. Initiation of shoot bud	93
4.1.2. Somatic embryogenesis and plantlet regeneration	95
4.1.3. Rooting and hardening of regenerated plants	96
<b>4.2. Optimization of <i>Agrobacterium</i>- mediated transformation protocol</b>	97
4.2.1. Optimum concentration of <i>Agrobacterium</i> culture, infection time and co-cultivation period	97
4.2.2. Efficacy of antibiotics on the growth of <i>Agrobacterium</i> and plant regeneration	98
4.2.3. Selection and regeneration of putative transgenics	98
<b>4.3. Molecular confirmation of T<sub>0</sub> sorghum transgenic plants</b>	106
4.3.1. PCR analysis of sorghum transgenic plants in T <sub>0</sub> generation	106
4.3.2. Southern blot analysis of sorghum transgenic plants in T <sub>0</sub> generation	106
<b>4.4. Molecular analysis of T<sub>1</sub> sorghum transgenic plants</b>	112
4.4.1. Inheritance of T <sub>1</sub> sorghum transgenic plants	112
4.4.2. Southern blot analysis T <sub>1</sub> sorghum transgenic plants	112
<b>4.5. Molecular analysis of T<sub>2</sub> sorghum transgenic event-A4</b>	120
4.5.1. PCR analysis	120
4.5.2. RT PCR analysis	120
<b>4.6. Insect bioassays of sorghum transgenic plants</b>	124
4.6.1. Leaf disc bioassay of sorghum transgenic eventA4	124
4.6.2. Whole plant bioassay sorghum transgenic eventA4	127
<b>5. DISCUSSIONS</b>	132
<b>5.1. <i>In vitro</i> regeneration system in sorghum</b>	132
<b>5.2. Genetic transformation in sorghum</b>	137
<b>5.3. <i>Agrobacterium</i>-mediated genetic transformation</b>	138
5.3.1. Selection and regeneration of putative transgenics	142
<b>5.4. Molecular analysis</b>	143
<b>5.5. Insect bioassays</b>	145
5.5.1. Leaf disc bioassay of plant progenies of transgenic event-A4	145
5.5.2. Whole plant bioassay of plant progenies of transgenic event-A4	146
<b>6. SUMMARY AND CONCLUSIONS</b>	148
<b>7. BIBLIOGRAPHY</b>	155
<b>8. APPENDIX</b>	198