

Copyright is owned by the Author of the thesis. Permission is given for a copy to be downloaded by an individual for the purpose of research and private study only. The thesis may not be reproduced elsewhere without the permission of the Author.

**INVESTIGATION OF THE POSSIBILITY OF INTROGRESSION FROM**  
*Trifolium ambiguum* M. Bieb. INTO *T. repens* L.

**A thesis submitted in the partial fulfilment of the requirements for the degree of**

**DOCTOR OF PHILOSOPHY**

**IN**

**PLANT BREEDING AND GENETICS**

**In the Institute of Fundamental Sciences, Massey University,**

**Palmerston North, NEW ZEALAND**

**By**

**IHSAN ULLAH**

**2013**



## ABSTRACT

The objective of this project was to investigate the possibility of introgression of stress resistance traits from *T. ambiguum* (A) into *T. repens* (R) by interspecific hybridisation, using two approaches. The first used *T. occidentale* Coombe (O) as a genetic bridge because this species has chromosome pairing homology to both the two other species. The second approach attempted direct integration of genomes from the two species through ploidy manipulation.

For the first approach, four crossing strategies used *T. occidentale* as a genetic bridge. Each started with different multispecies hybrids with various genomic contributions from the three parental species. The second approach began with 5x and 7x *T. ambiguum* x *T. repens* hybrids (ARRRR and AAARRRR). All the initial hybrids were repeatedly selfed, inter-crossed and backcrossed with colour-marked white clover and advanced progenies with reasonable levels of fertility were obtained in every strategy. Advanced hybrids were selected on the basis of flow cytometric ploidy estimation, phenotypes and somatic chromosome counts and were characterised for chromosome pairing and introgression events using both conventional and molecular cytogenetics. The advanced hybrid progenies were also grown in a sandpit to determine the relative expressions of the parental traits.

Chromosome analyses showed evidence of chromosome elimination, chromosome addition/substitution, allosyndetic pairing involving A-derived chromosomes and inter-specific genomic recombination. GISH analysis revealed that the genetic bridge strategy 1, which started with RRAO, gave a plant with four apparently large A-R and A-O chromosomal exchanges along with an A chromosome addition and a O-R substitution. No apparent signs of introgression were detected by GISH in the other strategies but introgression could not be ruled out because the sample was small and the morphology of the hybrids tested in the sandpit showed the expression of characters from both the parental species. These plants need to be characterised by using more genetic markers. The advanced progeny in the strategy based on direct integration of AxR genomes showed a low level of inter-specific chromosome pairing consistent with an absence of, or very low level of, introgression. The introgression revealed by GISH in the advanced progeny of RRAO hybrids provides evidence that using *T. occidentale* as genetic bridge

has worked by disrupting the genomic integrity in *T. repens*. The material having introgression has many applications from white clover improvement point of view. While direct hybrids with A & R genomes did not lead to any apparent introgression.

## ACKNOWLEDGEMENTS

### **In the name of Allah, Most Gracious, Most Merciful**

It is a great pleasure to appreciate and acknowledge the help I got, in one way or the other, from many people especially my supervisors, colleagues, friends and family members throughout this project right from start to end.

I appreciate my immediate advisor Prof. Warren Williams (Principal Scientist, Forage Improvement, Grasslands Research Centre, AgResearch and Professor of Plant Breeding in the Institutes of Fundamental Sciences, Massey University, New Zealand) for his outstanding help throughout this project right from the planning through execution to thesis writing. He has been very kind in taking out time and checking the manuscript critically in a timely way. I always benefited from his constructive criticism on my work, discussions and his ability for argument development and above all his moral and financial support throughout this endeavour.

I also offer my sincere thanks to my second supervisor, Prof. Micheal McManus, Institute of fundamental sciences, Massey University for his moral and financial help during the project. He has been very kind and helped in every way in making this project a success.

I am also thankful to Dr. Helal Ansari for his help with the cyto-molecular work. All the genomic *in situ* hybridization and fluorescent *in situ* hybridization work was done at his lab. He provided outstanding help and support throughout this huge work and would be remembered forever. I would also appreciate Dr. Syed Wajid Hussain for his help and support with the hybridization techniques, conventional cytology and field data collection. I also appreciate Dr. Nick Ellison for his help with DNA extraction and probe labelling. He has been outstanding throughout and deserves special thanks.

I would express my appreciation to Isabelle Williams for providing the original BAR hybrids and technical help throughout this study. Special thanks go to my friend Mr. M. Naeem for his help with miscellaneous things as and when needed. I would also appreciate the help of John Koolaard and Dongwen Luo for their help as statisticians with designing the field experiments, collecting and analysing the data. I also acknowledge Catherine Lloyd-West for her help with computer work.

I appreciate the Higher Education Commission, Pakistan, for the financial support for this research project. Last but not the least, I highly appreciate the valuable support I got from my mother, wife, mother-in law, brothers, sisters and three cute daughters during my PhD study and I dedicate this thesis to all of them. May Allah bless them with eternal happiness.

## TABLE OF CONTENTS

<b>CHAPTER 1</b>	<b>Page</b>
<b>INTRODUCTION</b>	<b>1</b>
<b>1.1 Background of the project.</b>	<b>4</b>
<b>1.2 Approach 1</b>	<b>6</b>
<b>1.2.1 Strategy 1</b>	<b>7</b>
<b>1.2.2 Strategy 2</b>	<b>8</b>
<b>1.2.3 Strategy 3</b>	<b>8</b>
<b>1.2.4 Strategy 4</b>	<b>9</b>
<b>1.3 Approach 2</b>	<b>10</b>
<b>1.3.1 Strategy 5.1</b>	<b>10</b>
<b>1.3.2 Strategy 5.2</b>	<b>11</b>
<b>1.4 Summary of the aims of this thesis</b>	<b>12</b>
<b>CHAPTER 2</b>	
<b>2.1 LITERATURE REVIEW</b>	<b>13</b>
<b>2.1.1 Wide crosses in germplasm improvement</b>	<b>13</b>
<b>2.2.1 Introduction to and significance of white clover (<i>T. repens</i> L.)</b>	<b>14</b>
<b>2.2.2 Cytogenetic description of <i>T. repens</i></b>	<b>15</b>
<b>2.2.3 Why <i>T. repens</i> needs agronomic improvement</b>	<b>16</b>
<b>2.3.1 <i>T. ambiguum</i> M. Bieb. as a novel source of variation for     <i>T. repens</i></b>	<b>18</b>
<b>2.4.1 <i>T. occidentale</i> as a genetic bridge</b>	<b>20</b>
<b>2.5.1 Wide hybridization in <i>Trifolium</i></b>	<b>21</b>
<b>2.5.1.1 Hybrids between <i>T. nigrescens</i> and <i>T. occidentale</i></b>	<b>22</b>
<b>2.5.1.2 Hybrids between <i>T. ambiguum</i> and <i>T. occidentale</i></b>	<b>23</b>
<b>2.5.1.3 Hybrids between <i>T. repens</i> and <i>T. nigrescens</i></b>	<b>24</b>
<b>2.5.1.4 Hybrids between <i>T. ambiguum</i> and <i>T. repens</i></b>	<b>25</b>
<b>2.5.2 Problems associated with wide crosses and their solution</b>	<b>31</b>
<b>2.5.3 Endosperm balance number (EBN) and inter-specific     crosses in <i>Trifolium</i></b>	<b>33</b>
<b>2.5.4 Meiotic abnormalities in hybrid situations</b>	<b>34</b>

2.6.1	Use of cyto-molecular tools in the characterization of hybrids	35
2.7.1	Genomic consequences of inter-specific hybridization and chromosome doubling	37
2.7.2	Gene dosages and expression levels	40
2.7.3	Advantages of genomic changes	40
2.8.1	Flow cytometric analysis of DNA content in hybrids	41
2.9.1	Control of chromosome pairing and the role of the wheat <i>Ph1</i> gene	42
2.9.2	Mechanism of action of the <i>Ph1</i> gene	44
2.9.3	The significance of <i>Ph1</i> in cytogenetics, breeding and evolution	45
2.9.4	Lack of chromosomes pairing leads to sterility	45
<b>CHAPTER 3</b>		
<b>MATERIALS AND METHODS</b>		
3.1	Crossing Techniques	47
3.2	Self-compatibility (SC) testing techniques	48
3.3	Pollen stainability	48
3.4	Seed germination and initial screening of the progeny	48
3.5	Flow cytometry-based ploidy analysis	49
3.6	Somatic chromosome preparation	49
3.7	Meiotic chromosome pairing analysis (conventional cytology)	51
3.8	Enzyme macerated meiotic chromosome preparation	51
3.9	Giemsa staining	52
3.10	Molecular cytogenetic analysis of hybrids	52
3.10.1	DNA probes and labelling for FISH and GISH	52
3.10.2	Fluorescence <i>in situ</i> hybridization	53
3.11	Morphological characterization of BAR09 and BAR10 hybrids	54
3.12	Analysis of variance, ANOVA	56
<b>CHAPTER 4</b>		
<b>RESULTS</b>		
4.1	Strategy 1: (using <i>T. occidentale</i> as a genetic bridge to combine	61

4x <i>T. ambiguum</i> and <i>T. repens</i> genomes)	61
4.1.1 Hybrids from the cross, RRRR x BL (AAOO) - RRAO (4x)	61
4.1.2 Progeny of the original BAR hybrids (BAR09 hybrids)	61
4.1.2.1 Progeny of cross, RRAO (4x) x RRRR and selfing of RRAO (4x) hybrids	61
4.1.2.2 Chromosome pairing analysis in BAR09-120	64
4.1.3 Self and cross progeny of the BAR09 hybrids (BAR10 hybrids)	66
4.1.3.1 Cross and self progeny of RRR(A <sub>4</sub> O <sub>4</sub> ) plants	66
4.1.3.2 Conventional chromosome pairing analysis in BAR10-126	67
4.1.4 Molecular cytogenetic analyses of BAR10-126	69
4.1.5 Morphological description	72
4.1.5.1 Morphological characterization of self and cross progeny of RRAO hybrids	72
4.1.5.2 Morphological description of the self and cross progeny of RRR(A <sub>4</sub> O <sub>4</sub> )	72
 4.2 Strategy 2: (using <i>T. occidentale</i> as genetic bridge to combine 4x <i>T. ambiguum</i> genomes with <i>T. repens</i> )	 78
4.2.1 Plants derived from crosses, 434-1 (AAO) x BN or BL (AAOO) - AAA OO (5x) and AAORR (5x)	78
4.2.2 Progeny of the original BAR hybrids (BAR09 hybrids)	80
4.2.2.1 Progeny of the crosses, AAA OO (5x) x RRRR and AAORR (5x) x RRRR	80
4.2.2.2 Chromosome pairing analysis in BAR09-97, BAR09-98, BAR09-106 and BAR09-110	80
4.2.3 Self and cross progeny of the BAR09 hybrids (BAR10 hybrids)	84
4.2.3.1 The self and cross progeny of RRAO(A <sub>4</sub> ) (~ 4.5x), AAA OO and RRRA(O <sub>4</sub> )	84
4.2.3.2 Meiotic chromosome analysis in BAR10-111	87
4.2.3.3 Meiotic chromosome analysis in BAR10-124	87
4.2.4 Molecular cytogenetic analysis of BAR10-111	88
4.2.5 Phenotypic characterization of hybrids	89

4.2.5.1	Self and cross progeny of 5x AAAOO with white clover	89
4.2.5.2	Self and cross progeny of RRAO(A <sub>4</sub> ) with white clover	91
4.3	Strategy 3: (using <i>T. occidentale</i> as genetic bridge to combine 6x <i>T. ambiguum</i> genomes with <i>T. repens</i> )	96
4.3.1	Hybrids derived from Hybrid 33 OP-1 – RRRA(A <sub>4</sub> O <sub>4</sub> ) (~5x) and RRR(R <sub>4</sub> A <sub>6</sub> O <sub>2</sub> ) (~4.5x)	96
4.3.2	Progeny of the original BAR hybrids (BAR09 hybrids)	99
4.3.2.1	Progeny of RRRA(A <sub>4</sub> O <sub>4</sub> ) (5x) and RRR(A <sub>6</sub> R <sub>4</sub> O <sub>2</sub> ) (4.5x) with <i>T.repens</i>	99
4.3.2.2	Meiotic chromosome pairing analysis in BAR09-62, BAR09-63 and BAR09-65	99
4.3.3	Self and cross progeny of the BAR09 hybrids (BAR10 hybrids)	103
4.3.3.1	Progeny of RRR(R <sub>4</sub> A <sub>6</sub> O <sub>2</sub> ) ~ 4.5x and RRR(R <sub>6</sub> A <sub>3</sub> O <sub>1</sub> ) ~ 4.25x	103
4.3.3.2	Meiotic chromosome analysis in BAR10-80, BAR10-81 and BAR10-93	103
4.3.4	Molecular cytogenetic analysis of BAR09-63, BAR10-81 and BAR10-93	107
4.3.4.1	Meiotic chromosome pairing analysis in BAR09-63	107
4.3.4.2	Genomic composition analysis in BAR09-63, BAR10-81 and BAR10-93	108
4.3.5.	Phenotypic studies of BAR09 and BAR10 hybrids	110
4.3.5.1	Self and cross progeny of 5x RRRA(A <sub>4</sub> O <sub>4</sub> ) and 4.5x RRR(R <sub>4</sub> A <sub>6</sub> O <sub>2</sub> )	110
4.3.5.2	Self and cross progeny of RRR(R <sub>4</sub> A <sub>6</sub> O <sub>2</sub> ) and RRR(R <sub>6</sub> A <sub>3</sub> O <sub>1</sub> )	111
4.4	Strategy 4: (inserting <i>T. occidentale</i> as a genetic bridge)	117
4.4.1	Hybrids derived from ROS (A <sup>T</sup> A <sup>T</sup> RR) x <i>T. occidentale</i> - ARO (3x) and AARROO (6x)	117
4.4.2	Progeny of the original BAR hybrids (BAR09 hybrids)	119
4.4.2.1	BAR09 progeny of the crosses, ARO (3x) x RRRR and AARROO (6x) x RRRR	119
4.4.2.2	Meiotic analysis of BAR09-3	119

4.4.3	Self and cross progeny of the BAR09 hybrids (BAR10 hybrids)	121
4.4.3.1	Progeny of RRRAO x RRRR- RRR(R <sub>4</sub> A <sub>4</sub> O <sub>4</sub> )	121
4.4.3.2	Meiosis in BAR10-1 and BAR10-22	123
4.4.4	Molecular cytogenetic analysis of BAR10-22	124
4.4.5	Phenotypic description of selected hybrids	125
4.4.5.1	Progeny of ARO (3x) and AARROO (6x) hybrids	125
4.4.5.2	Self and cross progeny of RRRAO (5x) hybrids	126
4.5	Strategy 5: (direct integration of R and A genomes through ploidy manipulation)	131
4.5.1	Hybrids from (A <sup>D</sup> A <sup>T</sup> RR, Hybrid-70) x (RRRR) - RRRRA (5x) and AAARRRR (7x)	131
4.5.2	Progeny of the original BAR hybrids (BAR09 hybrids)	133
4.5.2.1	Self and cross progeny of (5x) BAR hybrids, ARRRR with RRRR	133
4.5.2.2	Meiotic chromosome pairing analysis in BAR09-16, BAR09-19 and BAR09-24	133
4.5.3	Progeny of BAR09 hybrids (BAR 10 hybrids)	137
4.5.3.1	Progeny of cross RRRR(A <sub>4</sub> ) x RRRR- RRRR(A <sub>2</sub> )	137
4.5.3.2	Meiotic analysis of selected BAR10 hybrids	140
4.5.4	Molecular cytogenetic analysis of BAR09-16 and BAR10-32	141
4.5.5	Morphological characterization	143
4.5.5.1	Self and cross progeny of 5x RRRRA hybrids	143
4.5.5.2	Self and cross progeny of RRRR(A)	144
<b>CHAPTER 5</b>		
<b>DISCUSSION</b>		
5.1	Original BAR hybrids	150
5.2	Strategy based on 4x BAR hybrids, RRAO	151
5.2.1	Meiosis in BAR09-120 (2n=4x=33)	154
5.2.2	Meiosis in BAR10-126 (2n=4x=33)	155
5.2.3	Molecular cytogenetic analysis of BAR10-126	157

5.2.4	Morphology of RRR(A <sub>4</sub> O <sub>4</sub> ) (BAR09 hybrids) and RRR(R <sub>4</sub> A <sub>2</sub> O <sub>2</sub> ) (BAR10 hybrids)	158
5.3	Strategy based on 5x BAR hybrids having 3 As and 2 Os (AAA00)	160
5.3.1	Meiosis in BAR09 hybrids derived from cross, AAA00 (5x) x RRRR	164
5.3.1.1	Meiosis in BAR09-98	164
5.3.1.2	Meiosis in BAR09-110	164
5.3.1.3	Meiosis in BAR10-111	165
5.3.1.4	Meiosis in BAR10-124	166
5.3.2	Genomic composition analysis of BAR10-111	166
5.3.3	Morphology of hybrids derived from cross, AAA00 x RRRR – RRAO(A <sub>4</sub> )	168
5.3.4	Morphology of hybrids derived from cross, RRAO(A <sub>4</sub> ) x RRRR – RRR(A <sub>6</sub> O <sub>2</sub> )	168
5.4	Strategy using hybrid “33 OP-1, AAAORR” as starting material	169
5.4.1	First self and backcross progeny of hybrids, RRRR(A <sub>4</sub> O <sub>4</sub> ) or RRR(R <sub>4</sub> A <sub>6</sub> O <sub>2</sub> )	170
5.4.2	Second self and backcross progeny of hybrids, RRRR(A <sub>4</sub> O <sub>4</sub> ) or RRR(R <sub>4</sub> A <sub>6</sub> O <sub>2</sub> )	171
5.4.3	Chromosome pairing in BAR09-62, BAR09-63 and BAR09-65	172
5.4.4	Meiotic analysis of BAR10-80, BAR10-81 and BAR10-93	174
5.4.5	GISH/FISH on hybrid “33, AAAO” derived adv. progeny	175
5.4.5.1	GISH on BAR09-63	175
5.4.5.2	GISH on BAR10-81	176
5.4.5.3	GISH on BAR10-93	177
5.4.6	Morphology of RRRR(A <sub>4</sub> O <sub>4</sub> ) (5x), RRR(R <sub>4</sub> A <sub>6</sub> O <sub>2</sub> ) (4.5x) and RRR(R <sub>6</sub> A <sub>3</sub> O <sub>1</sub> ) (4.25x)	178
5.5	Strategy based on ARO (3x) and AARROO (6x)	179
5.5.1	Characterization of the first self and backcross progeny of ARO and AARROO hybrids	180

5.5.2	Characterization of the second self and backcross progeny of ARO and AARROO hybrids	182
5.5.3	Chromosome pairing analysis in BAR09-3 (RRRAO, 5x)	183
5.5.4	Meiotic analysis of BAR10-1(RRR(R <sub>4</sub> A <sub>4</sub> O <sub>4</sub> )) and BAR10-22 (RRR(R <sub>4</sub> A <sub>4</sub> O <sub>4</sub> ))	184
5.5.5	Molecular cytogenetic analysis of BAR10-22	186
5.5.6	Phenotypic characterization of hybrids from crosses, ARO x RRRR and AARROO x RRRR - RRRAO (5x)	186
5.5.7	Phenotypic description of the second self and cross progeny of RRRAO (5x) - RRR(R <sub>4</sub> A <sub>4</sub> O <sub>4</sub> )	188
5.6	Strategy based on the direct integration of A and R genomes-ARRRR (5x) and AAARRRR (7x) hybrids	189
5.6.1	The first self and backcross progeny of ARRRR and AAARRRR hybrids	190
5.6.2	The second self and backcross progeny of 5x hybrids, ARRRR	191
5.6.3	Meiosis in the hybrids of first self and cross progeny of 5x hybrids (ARRRR)	192
5.6.4	Meiotic chromosome behaviour in the BAR10 progeny of 5x hybrids, ARRRR	193
5.6.5	Molecular cytogenetic studies of analysis of BAR09-16 and 32	194
5.6.6	Morphology of hybrids derived from ARRRR x RRRR - RRRR(A)	196
	<b>RESULTS SUMMARY</b>	<b>199</b>
	<b>REFERENCES</b>	<b>203</b>

## LIST OF TABLES

Table	page
3.1	56
3.2	57
3.3	58
3.4	59
3.5	60
4.1.1	62
4.1.2	63
4.1.3	65
4.1.4	68
4.1.5	68
4.1.6.1	74
4.1.6.2	75
4.1.7.1	76
4.1.7.2	77
4.2.1	79
4.2.2	81
4.2.3	82
4.2.4	85
4.2.5	86
4.2.6.1	92

4.2.6.2	Mean morphological data of the above and below-ground traits of the progeny of the cross AAAOO x RRRR	93
4.2.7.1	Mean morphological data of the above-ground traits of the advanced progeny of the cross involving RRAO(A)	94
4.2.7.2	Mean morphological data of the above and under-ground traits of the advanced progeny of the cross involving RRAO(A) hybrids	95
4.3.1.1	Pedigrees, pollen fertilities, flow cytometric derived ploidy estimates and further crossing details of the original 5x hybrids, RRRRA(A <sub>4</sub> O <sub>4</sub> )	97
4.3.1.2	Pedigrees, pollen fertilities, flow cytometric derived ploidy estimates and further crossing details of the original 4.5x BAR hybrids, RRR(R <sub>4</sub> A <sub>6</sub> O <sub>2</sub> )	98
4.3.2	Selected progeny plants of original BAR hybrids, RRRRA(A <sub>4</sub> O <sub>4</sub> ) and RRR(R <sub>4</sub> A <sub>6</sub> O <sub>2</sub> ) (Tables 4.3.1.1, and 4.3.1.2)	100
4.3.3	Meiotic chromosome associations at diakinesis/metaphase-I in PMCs of BAR09-62, BAR09-63 and BAR09-65	101
4.3.4	Selected progeny plants of the BAR09 hybrids with expected genomic formula RRR(R <sub>4</sub> A <sub>6</sub> O <sub>2</sub> ) and RRR(R <sub>6</sub> A <sub>3</sub> O <sub>1</sub> )	104
4.3.5	Meiotic chromosome associations at diakinesis/metaphase-I in PMCs of BAR10-80, BAR10-81 and BAR10-93	105
4.3.6.1	Phenotyping of the advanced generation of the cross involving 6x 33-OP-1 (AAAORR) and white clover (RRRR)	112
4.3.6.2	Mean data of different above and under-ground morphological characters of the advanced generation of the cross involving 6x 33-OP-1 (AAAORR) and white clover (RRRR)	113
4.3.7.1	Mean data of different above-ground morphological characters of the advanced generation of the cross RRR(RAO) x white clover	114
4.3.7.2	Mean data of different above- and under-ground morphological characters of the advanced generation of the cross, RRR(RAO) x RRRR	115
4.4.1	Pedigrees of 3x (ARO) and 6x (AARROO) BAR hybrids derived from the cross of ROS (AARR, 4x) with <i>T. occidentale</i> (OCD = 2x and OCT= colchicine doubled 4x)	118
4.4.2	Progeny of ARO (3x) and AARROO (6x) hybrids (Table 4.4.1) with pedigrees, expected genomic composition and estimated ploidies	120
4.4.3	Meiotic chromosome associations at diakinesis/metaphase-I in PMCs of BAR09-3 (RRRAO, 2n=40)	120
4.4.4	Selected progeny of the BAR09 hybrids with genomic formula RRRRAO (Table 4.4.2)	122
4.4.5	Meiotic chromosome associations at diakinesis/metaphase-I in PMCs of hybrid BAR10-1 (2n=35) and BAR10-12 (2n=33)	122
4.4.6.1	Mean data of above-ground morphological data of the advanced progeny of the crosses of 3x ARO and 6x AARROO hybrids	127
4.4.6.2	Mean data of above and under-ground morphological data of the advanced progeny of the crosses of 3x ARO and 6x AARROO	128
4.4.7.1	Mean data of above-ground morphological traits of the advanced progeny of the cross of RRRRAO (5x) with white clover (RRRR)	129
4.4.7.2	Mean data of above- and under-ground morphological traits of the advanced progeny of the cross of RRRRAO x RRRR	130
4.5.1	Pedigrees of 14 nearly 5x (ARRRR) and 7x (AAARRRR) hybrids with pollen fertility, flow cytometric ploidy estimates	132
4.5.2	Selected progeny plants of original hybrids RRRRA (5x) (Table 4.5.1) with their expected genome composition, pollen fertilities	134

4.5.3	Meiotic chromosome associations at diakinesis/metaphase-I in PMCs of BAR09-16, BAR09-19 and BAR09-24	135
4.5.4	Selected progeny of the BAR09 hybrids with expected genomic formula RRRR(A <sub>4</sub> ) (4.5x) and RRRRA (5x) (Table 4.5.2), with ploidy estimates (flow cytometry), expected and actual chromosome counts	138
4.5.5	Meiotic chromosome associations at diakinesis/metaphase-I in PMCs of BAR10-39, BAR10-49, BAR10-58, BAR10-59 and BAR10-63	139
4.5.6.1	Data on the morphological characteristics of the self and cross progeny of 5x BAR hybrids (RRRRA) with white clover (RRRR)	145
4.5.6.2	Data on different above- and under-ground morphological characteristics of the self and cross progeny of 5x BAR hybrids, ARRRR	146
4.5.7.1	Mean data of above-ground morphological characters of the progeny of the cross between 4.5x hybrids RRRR(A <sub>4</sub> ) and white clover	147
4.5.7.2	Mean data of above and under-ground morphological characters of the progeny of the cross between 4.5x hybrids RRRR(A) and RRRR	148

## LIST OF FIGURES

Figure	page
4.1.1 Giemsa-stained mid-metaphase chromosomes in BAR09-120 (RRR(A <sub>4</sub> O <sub>4</sub> )+1, 2n= 33).	66
4.1.2 Giemsa-stained early metaphase chromosomes in BAR10-126 (RRR(R <sub>4</sub> A <sub>2</sub> O <sub>2</sub> )+1, 2n=33)	67
4.1.3 DAPI-stained (grey scale) metaphase chromosomes in BAR10-126 (RRR(R <sub>4</sub> A <sub>2</sub> O <sub>2</sub> )+1, 2n=33).	70
4.1.4 GISH/FISH on the meiotic chromosomes of BAR10-126	71
4.2.1 Giemsa-stained early-to- mid metaphase chromosomes in BAR09-98 (RRAO(A <sub>4</sub> )-2, 2n=34). Three satellite knobs are visible (arrows); two are small and similar in size while the third is larger and is possibly from <i>T.ambiguum</i> . The <i>T. ambiguum</i> -derived chromosomes	83
4.2.2 Geimsa-stained somatic chromosomes in BAR10-111 (RRR(A <sub>6</sub> O <sub>4</sub> )-1, 2n=35) with 3 satellite knobs (arrows) one of which is comparatively bigger	88
4.2.3 GISH-FISH on the mitotic chromosomes of BAR10-111	89
4.3.1 Giemsa-stained somatic chromosomes in BAR09-63 (RRR(R <sub>6</sub> A <sub>3</sub> O <sub>1</sub> )-1, 2n=33). Two satellite knobs are seen lying away from the main chromosomal bodies (arrows)	102
4.3.2 Giemsa-stained somatic chromosomes in BAR10-81 (RRR(R <sub>7</sub> A <sub>1-2</sub> O <sub>0-1</sub> ), 2n=33) having two satellite knobs (arrows) lying away from the main chromosomes	106
4.3.3 Giemsa-stained somatic and meiotic chromosomes analysis in BAR10-93	107
4.3.4 GISH-FISH on a meiotic chromosome spread in BAR09-63 using gDNA of <i>T. ambiguum</i> labelled with Fluor-X-dCTP (green) and Cy3-dCTP labelled pTr5S (red) as probes	108
4.3.5 GISH-FISH on somatic chromosome preparations of BAR09-63 (a-c), BAR10-81 (d-f) and BAR10-93 (g-i)	109
4.4.1 Metaphase-I in BAR09-3 (RRRAO, 2n=40) with Is, IIs, IIIs and IVs (arrows). (b) Highly disturbed anaphase-I in BAR09-3 with several chromosomes lagging behind	121
4.4.2 Giemsa-stained somatic chromosomes in BAR10-22 (2n=33) with two chromosomes having satellite knobs	124

<b>4.4.3</b>	<b>GISH and FISH on metaphase chromosomes of BAR10-22</b>	<b>125</b>
<b>4.5.1</b>	<b>BAR09-16 (RRRR(A<sub>4</sub>), 2n=36) (a). Giemsa-stained somatic chromosomes showing 36 chromosomes in early metaphase</b>	<b>136</b>
<b>4.5.2</b>	<b>Giemsa-stained chromosomes in BAR10-32 (RRRR(A), 2n=36) showing two satellite knobs (arrows)</b>	<b>141</b>
<b>4.5.3</b>	<b>GISH and FISH on somatic chromosomes in BAR09-16 and BAR10-32</b>	<b>142</b>

## ABBREVIATIONS AND TERMINOLOGY

The following abbreviations and terminology were used:

$\mu l$	microlitre
33	designation for 4x hybrid between 6x <i>T. ambiguum</i> and 2x <i>T. occidentale</i> (AAAO).
434-1	designation for 3x hybrid between 4x <i>T. ambiguum</i> and 2x <i>T. occidentale</i> (AAO).
AA	acetic acid
AFLP	amplified fragment length polymorphism
AMV	alfalfa mosaic virus
BAR	Bridging <i>Ambiguum Repens</i> .
BAR09	the first progeny of original BAR hybrids bred and grown in 2009.
BAR10	the second progeny of original BAR hybrids bred and grown in 2010.
BC	backcross
BL	a 4x hybrid between between 4x <i>T. ambiguum</i> and 4x <i>T. occidentale</i> (AAOO).
BN	a 4x hybrid between between 4x <i>T. ambiguum</i> and 4x <i>T. occidentale</i> (AAOO).
CBC	congruity backcross
cm	centimetre
CYVV	clover yellow vein virus
DAPI	4',6-diamidino-2-phenylindole.
DNA	deoxyribonucleic acid
EBN	endosperm balance number
F <sub>1</sub>	first filial generation
F <sub>2</sub>	second filial generation
FC	flow cytometry

FISH	fluorescence <i>in situ</i> hybridization
GISH	genomic <i>in situ</i> hybridization
H-435	hybrid 435 with genomic composition, A <sup>T</sup> A <sup>T</sup> RR (4x)
Hybrid 70	designation for 4x hybrid, A <sup>D</sup> A <sup>T</sup> RR with two <i>T. ambiguum</i> genomes coming from diploid and tetraploid sources.
IIIs	trivalents
IIs	bivalents
Is	univalent chromosomal associations during anaphase-I
ITS	internal transcribed spacer
IVs	quadrivalents
MPV	mid parental value
mRNA	messenger RNA
MSAP	methylation sensitive amplification polymorphism
N	nitrogen
<i>ng</i>	Nanograms
NOR	nucleolar organizer region
OP	open-pollinated
<i>Ph1</i>	pairing homoeologous 1
PI	Propidium iodide
PMCs	pollen mother cells
PSV	peanut stunt virus
rDNA	ribosomal DNA
RET	<i>T. repens</i> with tetraploid genomic composition.
RNA	ribonucleic acid
RO	a white clover genotype named as Red One
ROS	designation for a 4x hybrid, AARR.
SC	self-compatible

SDR	second division restitution
SI	self-incompatible
SSC	saline sodium citrate
v/v	volume/volume
Vs	pentavalents
w/v	weight/volume
WCMV	white clover mosaic virus

**Terminology for identifying different genomes/sub-genomes in different species**

A	one sub-genome (x=8) from <i>T. ambiguum</i> (origin unspecified)
A <sup>D</sup>	one sub-genome (x=8) from 2x <i>T. ambiguum</i>
A <sup>T</sup>	one sub-genome (x=8) from 4x <i>T. ambiguum</i>
A <sup>H</sup>	one sub-genome (x=8) from 6x <i>T. ambiguum</i>
O	one sub-genome (x=8) from 2x <i>T. occidentale</i>
R	one sub-genome (x=8) from white clover
R <sup>P</sup>	<i>T. pallescens</i> -derived subgenome of <i>T. repens</i>
R <sup>O</sup>	<i>T. occidentale</i> -derived subgenome of <i>T. repens</i>

**Partial and mixed sub-genomes are designated as follows:**

(A)	a partial sub-genome (x=unspecified number, 1-7) from <i>T. ambiguum</i>
(A <sub>4</sub> )	a partial sub-genome (x=4) from <i>T. ambiguum</i>
(R/A)	a mixed sub-genome (x~8) containing both white clover and <i>T. ambiguum</i> chromosomes.
(A/O)	a mixed sub-genome (x~8) containing both <i>T. occidentale</i> and <i>T. ambiguum</i> chromosomes
(R <sub>4</sub> /A <sub>4</sub> )	a mixed sub-genome (x=8) containing four white clover and four <i>T. ambiguum</i> chromosomes.
(AO)	partial sub-genomes (x = 1-7) from <i>T. ambiguum</i> and <i>T. occidentale</i> .
(RAO)	partial sub-genomes (x = 1-7) from all the three species, white clover, <i>T. ambiguum</i> and <i>T. occidentale</i> .

- A<sup>O</sup> a recombinant chromosome having *T. ambiguum* centromere with arms introgressed from *T. occidentale*
- O<sup>A</sup> a recombinant chromosome having *T. occidentale* centromere with arms introgressed from *T. ambiguum*.