Theor Appl Genet (1997) 95:678–690 © Springer-Verlag 1997

S. W. Hussain · W. M. Williams

Development of a fertile genetic bridge between *Trifolium ambiguum* M. Bieb. and *T. repens* L.

Received: 2 August 1996 / Accepted: 4 April 1997

Abstract *Trifolium ambiguum* M. Bieb and *T. repens* L. are taxonomically related but very difficult to cross. The rare hybrids so far reported between these two species were obtained only by embryo culture. This difficulty has been overcome in the present research by the creation of a "fertile bridge" between *T. ambiguum* and *T. repens*. Characters of interest can now be transferred from *T. ambiguum* to *T. repens* by using this "fertile bridge" without the use of sophisticated techniques. An array of backcross progenies was generated from crosses between a *T. ambiguum* × *T. repens* F_1 hybrid (8*x* H-435) and its parental species. The 8*x* hybrid was cross-fertile only with *T. repens* and resulted in 145 seeds from 1578 reciprocal crosses. Eleven of nineteen initially grown BC_1F_1 plants were all hexaploid with an average pollen stainability of 41.6%. A high frequency of multivalents at metaphase-I indicated that both autosyndetic and allosyndetic pairing occurred. Backcrosses of 6*x* BC_1F_1 plants to *T. repens* resulted in 5*x* BC_2F_1 plants with an average pollen stainability of 59.3%. On the other hand, 6*x* $BC_1F_1 \times 6x$ *T. ambiguum* crosses did not produce any seed and only two pentaploid plants were obtained from 6*x* $BC_1F_1 \times 4x$ *T. ambiguum* crosses. The difficulty encountered in generating 6*x* backcross progeny with 6*x* *T. ambiguum* was overcome by intercrossing the 6*x* BC_1F_1 plants and producing 6*x* BC_1F_2 plants with an average pollen stainability of 65.8%. One of these 6*x* BC_1F_2 plants was cross-compatible as a female with 6*x* *T. ambiguum* and resulted in CBC_2 plants that were all cross-compatible with 6*x* *T. ambiguum*. The 6*x* BC_1F_2 plants are likely to be superior to 6*x* BC_1F_1 progeny, as they have exhibited better expression of the combined rhizomatous and stoloniferous growth habit, improved fertility, more frequent nodal rooting and heavier nodulation. Consequently, the 6*x* BC_1F_2 plants can either be used directly in the selection programme or as a "fertile bridge" between the two parental species. The present work has resulted in the development of a series of fertile hybrids by the manipulation of chromosome numbers, combining the agronomic characteristics of the parent species in varying genome balances and at a range of ploidy levels. It is concluded that the initial sterility of the primary interspecific hybrids need not be a barrier to successful inter-breeding.

Key words Interspecific hybrids · *Trifolium repens* · *Trifolium ambiguum* · Rhizomes · Stolons

Communicated by H. F. Linskens

S. W. Hussain (✉) · W. M. Williams
AgResearch Grasslands, Private Bag 11008, Palmerston North, New Zealand

Introduction

White clover (*Trifolium repens* L. $2n = 4x = 32$) is one of the most important and widely planted forage legumes in temperate regions of the world. Although perennial, stands of white clover often decline significantly in the 2nd or 3rd year of growth due to the death of the taproot (Westbrook and Tesar 1955), susceptibility to a number of stress factors including drought (Bryant 1974; Spencer et al. 1975), various viral diseases (Barnett and Gibson 1975; McLaughlin and Pederson 1985; Alconero et al. 1986; Ragland et al. 1986), nematodes (Yeates et al. 1973; Skipp and Gaynor 1987; Mercer 1988; Pederson and Windham 1989), and other root-chewing insects (Gaynor and Skipp 1987). *T. ambiguum* M. Bieb, on the other hand, is tolerant to several viral diseases (Barnett and Gibson 1975; Jones et al. 1981; Pederson and McLaughlin 1989), spreads by means of underground rhizomes rather than aboveground stolons, and has the ability to persist under dry conditions due to its deep well-developed root and

rhizome system (Spencer et al. 1975). It is also one of the few species in the genus that has a naturally occurring ploidy series. Interspecific hybridization between this species and white clover therefore offers the potential for improvement of several weaknesses of white clover.

Although white clover has been successfully hybridised with *T. ambiguum* (Williams 1978; Williams and Verry 1981; Yamada and Fukuoka 1986; Yamada et al. 1989; Meredith et al. 1995) most of these crosses required embryo rescue, were obtained with difficulty, and the success rates were very low. At the time this project was initiated, only one partially fertile F_1 *T. ambiguum* × *T. repens* hybrid was available (Williams et al. 1990). This hybrid (designated 4*x* H-435) while only partially fertile was cross-fertile with the *T. repens* parent in backcrosses. H-435 has been chromosome-doubled (Anderson et al. 1991) and 8*x* H-435 was a little more fertile than the tetraploid clones, but again was cross-fertile only with *T. repens* and no confirmed backcross progeny was obtained with *T. ambiguum*. Therefore it remained uncertain whether the octoploid might be used as a "fertile bridge" between the two parental species.

Our first objective was to create a fertile bridging population between *T. repens* and *T. ambiguum*, preferably at a hexaploid level, by backcrossing octoploid H-435 to both of its parental species. As the two species are very difficult to cross, the creation of a "fertile bridge" would overcome the difficulty of crossing these two species and would also remove the need to derive further primary hybrids by using the sophisticated techniques of embryo rescue with nurse endosperm (Williams and Verry 1981) or ovule culture (Yamada et al. 1989). Once created, the fertile bridging plants at the hexaploid level could be efficiently used for transferring characters of interest from one species to another. The second objective of the current research was to estimate the extent of chromosome homology between *T. repens* and *T. ambiguum* by studying the pollen stainability and cytogenetics of F_1 and backcross populations.

Materials and methods

Eight plants of tetraploid *T. ambiguum* cv "Treeline" ($2n = 4x = 32$) and six plants of hexaploid *T. ambiguum* cv "Prairie" ($2n = 6x = 48$) were used. The ploidy level was confirmed for three tetraploid and two hexaploid plants using dry pollen shape (Taylor et al. 1976) and root-tip squashes. All *T. ambiguum* plants carried a white "V" leaf mark. One genotype of *T. repens* "Grasslands Crimson Charm" (CC-1) with red and "silver sprite" leaf-mark alleles in the heterozygous condition ($V^m V^i$; $R^l r$) was used. This plant also carried a multi-foliolate trait, probably also in heterozygous form. The expression of this character is variable. Three vegetatively propagated plants of 4*x* H-435 were obtained from AgResearch Grasslands, Palmerston North, New Zealand. C_0 clones of 8*x* H-435 were obtained from N. L. Taylor, Department of Agronomy, University of Kentucky (Taylor et al. 1991). Neither hybrid carried a leaf mark.

Pollination

Self-incompatibility of individual plants used in the crosses was assessed by gently rolling at least four bagged flower heads of each plant between the thumb and fingers every day for 3 days after bagging (Williams 1987). Because of the poor dehiscence of 4*x* H-435 the anthers of this plant were first ruptured with a forceps and the stigma carefully self pollinated with the pollen squeezed from the anthers. The flowers were protected from foreign pollen with paper bags.

Reciprocal crosses were made by hand on potted plants grown in the glasshouse. Before pollination, flowers on the female parent were emasculated by the forceps technique of Williams (1954).

Backcrossing schemes and terminology were adapted from Haghighi and Ascher (1988). The first backcross (BC_1F_1) involves 8*x* H-435 crossed with *T. repens* or *T. ambiguum*. The second backcross (BC_2F_1) involved one of the BC_1F_1 crossed with the recurrent parent. Congruity backcrosses (CBC) were also used in the present study. Here 4*x* and 8*x* H-435 were backcrossed to each of the parental species in alternate generations. For example, (8*x* H-435) was backcrossed with *T. repens*. This BC_1F_1 was then crossed with *T. ambiguum* to yield CBC_2 progeny. Progenies from $BC_1F_1 \times BC_1F_1$ intercrosses were designated BC_1F_2. The production of BC_1F_1 seeds was initiated in January 1992 and the crossing scheme was completed in January 1994.

Pod-development data were recorded as the total number of pods developed during the first 2 weeks after crossing. About 4–5 weeks after pollination, seeds were harvested from mature flower heads and stored for 4–6 weeks in a refrigerator at 4°C to break dormancy.

Cytological techniques

Pollen stainability was used to estimate pollen fertility. Two to three anthers from glasshouse-grown plants were dehisced over a glass slide to which a drop of 2% acetocarmine was added. After 5 min the percentage of plump, fully stained grains was determined. At least 1200 grains from six or more flowers and three or more inflorescences per plant were examined.

Somatic chromosome counts were made from root-tip squashes by an adaptation of the method of Williams (1978). Root tips were rinsed once with distilled water, pre-treated in 0.004 M 8-hydroxyquinoline for 5–7 h at 4°C and fixed 3 : 1 95% ethanol : glacial acetic acid at room temperature. The material was then rinsed twice with distilled water and hydrolysed in 1 N HCl at 60°C for 10–12 min and Feulgen stained for 15–30 min. Stained root tips were squashed in 2% acetocarmine for chromosome counts. At least ten cells from five root tips were examined for each plant.

Meiotic chromosome configurations were studied in pollen mother cells (PMCs) using a revision of the method of Giri et al. (1981). Young inflorescences (about 2 mm in diameter and just after emergence from the stipules) were fixed in Carnoy's fluid (6 : 3 : 1 95% ethanol : chloroform : glacial acetic acid) for 24 h at room temperature. Fixed flower buds were rinsed three times with 70% ethanol allowing at least 20 min for each change and stained in alcoholic hydrochloric acid-carmine stain (Snow 1963) for at least 72 h. After rinsing with 70% ethanol, the stained material was stored in 70% ethanol in the refrigerator until used. Anthers were squashed in warmed 1% acetocarmine on a glass slide and chromosomal associations were recorded at metaphase-I in 15–35 pollen mother cells from at least ten flower buds from each plant. Multivalent associations in many *Trifolium* species are often difficult to analyse with certainty (Williams et al. 1982) and so, in this study, first the total number of meiotic configurations was counted, then the numbers of definite univalents, bivalents, trivalents and quadrivalents were counted, and finally the uncertain associations, if any, were estimated on the basis of the total chromosome complement.

Seed-surface sterilisation and germination

Seeds from the first backcrosses and subsequent crosses were surface-sterilised by treatment for 30 s in 95% (v/v) ethanol which was then drained off and replaced with 0.2% (w/v) $HgCl_2$ acidified with 0.5% (v/v) HCl for 6 min, followed by five rinses with sterile water, and then placed in a shallow layer of sterile water overnight in a Petri dish at room temperature to imbibe. The seeds were scarified by the action of HCl during surface sterilisation. Surface-sterilised seeds were germinated on 0.8% (w/v) agar at 26°C under 10 μ Em^{-2} s^{-1} of light and a 16-h photoperiod. Two-week-old seedlings were then transferred to 10-cm plastic pots containing pasteurised potting mix and grown in the glasshouse. No inoculation with rhizobia was carried out and, under these circumstances, *T. repens* was always effectively nodulated and *T. ambiguum* non-nodulated by the common rhizobia in the environment.

Results

Self pollination of eight inflorescences (more than 400 flowers) of *T. repens* (CC-1), five inflorescences (more than 250 flowers) from each of the three plants of 4*x* *T. ambiguum* (cv Treeline) and three plants of 6*x* *T. ambiguum* (cv Prairie) resulted in no seed, thus confirming the self-incompatibility of these genotypes. Although 4*x* H-435 produced no seed after selfing ten inflorescences (more than 500 flowers) in the present investigation, its self-compatibility has previously been reported by Williams et al. (1982). Self-pollinations of 12 inflorescences (more than 700 flowers) of the colchicine-doubled (C_0) 8*x* H-435 resulted in 18 seeds from which two seeds were germinated and grown into mature plants.

First backcross (BC_1F_1)

Results from the reciprocal backcrosses of 4*x* and 8*x* H-435 to one genotype of *T. repens* (CC-1), three genotypes of 4*x* *T. ambiguum* (cv Treeline) and three genotypes of 6*x* *T. ambiguum* (cv Prairie) are presented in Table 1. Reciprocal backcrosses of 4*x* and 8*x* H-435 with tetraploid and hexaploid *T. ambiguum* were not successful. Only one seed was harvested from 4*x* H-435 as the female parent after pollination with *T. repens* (CC-1) pollen. The seed was germinated but failed to produce a healthy seedling and died after 10 days.

Fifteen mature plants were grown after germination of 17 of the 124 seeds harvested from 8*x* H-435 pollinated with *T. repens* (CC-1). All 15 BC_1F_1 plants carried leaf markings (Table 2, Fig. 1) derived from the *T. repens* (CC-1) male parent, thus confirming the backcross origin of the BC_1F_1 progeny. Twenty one fully developed seeds were obtained from *T. repens* (CC-1) after pollination with 8*x* H-435 pollen, from which four seeds were germinated and grown into mature plants. All 19 BC_1F_1 plants were self-incompatible as no seed

Table 1 Results of reciprocal first backcrosses of 4*x* and 8*x* H-435 to *T. ambiguum* (both 4*x* and 6*x*) and *T. repens*, second backcrosses (BC_2F_1), congruity backcrosses, $F_1 \times F_1$, and $BC_1F_1 \times BC_1F_1$ intercrosses (BC_1F_2)

Cross type	Number of crosses	Pod development		Number of seeds obtained
		No.	(%)	
First backcross (BC_1F_1)				
8*x* H-435 × CC-1	1320	683	51.7	124
CC-1 × 8*x* H-435	258	169	65.7	21
8*x* H-435 × 4*x* *T. ambiguum*	1054	7	0.7	0
4*x* *T. ambiguum* × 8*x* H-435	280	54	19.3	0
8*x* H-435 × 6*x* *T. ambiguum*	1032	0	0.0	0
6*x* *T. ambiguum* × 8*x* H-435	337	53	15.8	0
4*x* H-435 × CC-1	1079	288	26.6	1
CC-1 × 4*x* H-435	185	2	1.1	0
4*x* H-435 × 4*x* *T. ambiguum*	970	1	0.1	0
Second backcross (BC_2F_1)				
6*x* BC_1F_1 × CC-1	760	484	63.7	134
Congruity backcross (CBC_2)				
6*x* BC_1F_1 × 4*x* *T. ambiguum*	974	156	16.0	3
4*x* *T. ambiguum* × 6*x* BC_1F_1	178	0	0.0	0
6*x* BC_1F_1 × 6*x* *T. ambiguum*	830	284	34.2	0
6*x* *T. ambiguum* × 6*x* BC_1F	250	8	3.2	0
6*x* BC_1F_2 × 6*x* *T. ambiguum*[a]	318	163	51.3	17
6*x* *T. ambiguum* × 6*x* BC_1F_2[a]	41	185	44.8	0
Intercross (BC_2F_1)				
6*x* BC_1F_1 × 6*x* BC_1F_1	663	328	49.5	114
CBC_2 × 6*x* *T. ambiguum*	360	273	75.6	180
$F_1 \times F_1$				
4*x* H-435 × 8*x* H-435	1021	324	31.7	3

[a] Congruity backcross following $BC_1F_1 \times BC_1F_1$ intercross

Table 2 Percent pollen stainability of 4*x* and 8*x* H-435, first backcrosses (BC_1F_1), second backcrosses (BC_2F_1), congruity backcross (CBC_2), and $BC_1F_1 \times BC_1F_1$ intercrosses, and leaf markings in BC_1F_1 plants

Genotype	% Pollen stainability	Leaf marking[a]
Parents		
T. ambiguum (cv Treeline)	96.8	V^h-
T. ambiguum (cv Prairie)	98.3	V^h-
T. repens (CC-1)	90.5	$V^m V^i$; R^l-
4*x* H-435	13.3	*vv*
8*x* H-435	34.4	*vv vv*
BC_1F_1		
(8*x* H-435 × CC-1)-1	21.8	V^m-; *r*-
(8*x* H-435 × CC-1)-2	46.2	V^i-; R^l-
(8*x* H-435 × CC-1)-3	64.9	V^i-; *r*-
(8*x* H-435 × CC-1)-4	34.1	V^m-; R^l-
(8*x* H-435 × CC-1)-5	28.4	V^i-; R^l-
(8*x* H-435 × CC-1)-6	32.0	V^m-; R^l-
(CC-1 × 8*x* H-435)-1	19.1	V^m-; R^l-
(CC-1 × 8*x* H-435)-2	22.5	V^m-; *r*-
(CC-1 × 8*x* H-435)-3	26.9	V^i-; R^l-
$BC_1F_2(BC_1F_1 \times BC_1F_1)$		
[(CC-1 × 8*x* H-435)-2 × (CC-1 × 8*x* H-435)-3]-1	55.7	
[(CC-1 × 8*x* H-435)-2 × (CC-1 × 8*x* H-435)-3]-2	0.8	
[(CC-1 × 8*x* H-435)-2 × (CC-1 × 8*x* H-435)-3]-3	74.3	
[(CC-1 × 8*x* H-435)-2 × (CC-1 × 8*x* H-435)-3]-4	44.6	
[(CC-1 × 8*x* H-435)-1 × (CC-1 × 8*x* H-435)-2]-1	53.4	
[(CC-1 × 8*x* H-435)-1 × (8*x* H-435 × CC-1)-2]-1	16.1	
BC_2F_1		
[(CC-1 × 8*x* H-435)-2 × CC-1]-1	44.4	
[(CC-1 × 8*x* H-435)-2 × CC-1]-2	59.8	
[(CC-1 × 8*x* H-435)-2 × CC-1]-6	62.8	
[(CC-1 × 8*x* H-435)-2 × CC-1]-7	70.1	
Congruity backcrosses		
(8*x* H-435 × CC-1)-2 × 4*x T. ambiguum*	17.6	
(BC_1F_2 × 6*x T. ambiguum*)-1[b]	96.6	
(BC_1F_2 × 6*x T. ambiguum*)-2[b]	89.8	
(BC_1F_2 × 6*x T. ambiguum*)-3[b]	94.3	

[a] Leaf marking references: V^h, V^i (full V, high, intermediate, respectively), Brewbaker and Carnahan (1956); V^m (white tissue distal to the V marking), Lenoble and Papineau (1970); R^l (the upper leaf surface is dark purple and the lower surface is slightly coloured), Carnahan et al. (1955)

[b] Congruity backcross following $BC_1F_1 \times BC_1F_1$ intercross

was obtained from any plant after selfing at least eight inflorescences on each plant.

Congruity (CBC) and second (BC_2F_1) backcrosses

Two genotypes of BC_1F_1, (8*x* H-435 × CC-1)-2 and (CC-1 × 8*x* H-435)-1, were reciprocally backcrossed with one tetraploid (cv Treeline) and three hexaploid (cv Prairie) *T. ambiguum* genotypes. Significant pod development was observed during the first 2 weeks after pollination (Table 1) for crosses involving BC_1F_1 as the female parent. However, most pods failed to develop seeds. Only three seeds were harvested from (8*x* H435 × CC-1)-2 pollinated with 4*x T. ambiguum*. In reciprocal crosses of this combination, pod development was either absent or very poor. No seeds were harvested from these reciprocal crosses.

Two genotypes of BC_1F_1, (CC-1 × 8*x* H-435)-1 and (CC-1 × 8*x* H-435)-2, were employed in second backcrosses with *T. repens* (CC-1) using *T. repens* as a male parent only. Significant pod development was observed during the 1st and 2nd weeks following pollination (Table 1). Most of the pods contained shrunken non-germinable seeds, although approximately 17% of the developed pods had either one or two fully developed seeds. Reciprocal backcrosses of this combination were not attempted. Seven BC_2F_1 seeds were germinated and grown into mature plants.

$BC_1F_1 \times BC_1F_1$ intercrosses (BC_1F_2)

Three BC_1F_1 genotypes, (CC-1 × 8*x* H-435)-1, (CC-1 × 8*x* H-435)-2 and (8*x* H-435 × CC-1)-2, were intercrossed and produced 114 fully developed BC_1F_2 seeds

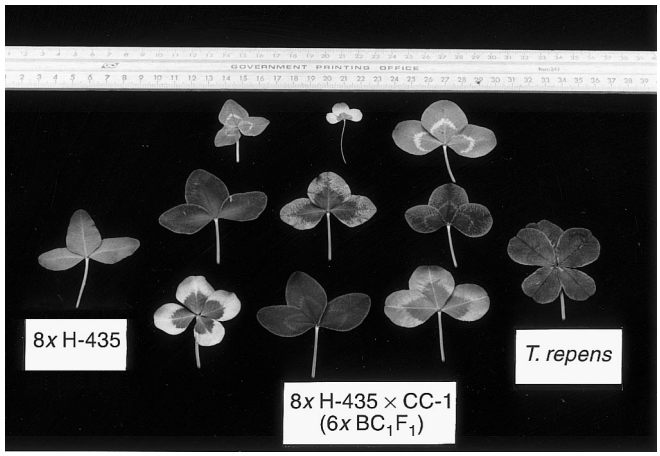

Fig. 1 Leaf markings in a 6*x* BC_1F_1 (8*x* H-435 × *T. repens*) derived from a *T. repens* male parent. Extreme left, 8*x* H-435 (*T. ambiguum* × *T. repens*) with no leaf mark; central three columns, 6*x* BC_1F_1 plants, all with leaf markings and one showing the multi-leaflet character; extreme right *T. repens* (CC-1) with red leaflets and multi-leaflet traits

from 663 crosses (Table 1). Six of these seeds were germinated and grown into mature plants. Four of the six initially grown BC_1F_2 plants exhibited combined stoloniferous and rhizomatous growth with frequent nodal rooting and nodulation (see Fig. 4). Upon flowering, two BC_1F_2 genotypes were reciprocally backcrossed with one genotype of 6*x T. ambiguum* (cv Prairie). Seventeen seeds were harvested from one BC_1F_2, [(CC-1 × 8*x* H-435)-2 × (CC-1 × 8*x* H-435)-3]-1, female parent. All three of the initially grown CBC_2 plants were cross compatible with 6*x T. ambiguum* and gave approximately 180 seeds from 360 crosses. Where 6*x T. ambiguum* was used as the female and BC_1F_2 plants as male parent, the crosses did not produce any seed, despite better early pod development in comparison to some earlier crosses where BC_1F_1s were used as male parents (Table 1). One of the six BC_1F_2 plants, [(CC-1 × 8*x* H-435)-2 × (CC-1 × 8*x* H-435)-3]-4, was self-compatible as it produced 161 seeds after self-pollination of nine inflorescences.

Three fully developed seeds were also obtained from a 4*x* H-435 × 8*x* H-435 cross using 4*x* H-435 as the female parent. Two fully developed plants were grown after germinating these seeds. The third seedling died about 2 weeks after germination.

Somatic chromosomes

The somatic chromosomes number for *T. repens* (CC-1) was confirmed as $2n = 4x = 32$ (Fig. 2). Three genotypes of 4*x T. ambiguum* (cv Treeline) and two genotypes of 6*x T. ambiguum* (cv Prairie) were also evaluated cytologically for somatic chromosome counts and, as expected, were found to be $2n = 4x = 32$ and $2n = 6x = 48$ respectively (Fig. 2).

Eight of fifteen BC_1F_1 plants obtained using 8*x* H-435 as the female parent and *T. repens* as the male parent were all found to be hexaploids with a somatic chromosome number of $2n = 6x = 48$ (Fig. 2). The remaining seven plants were not assessed. Three out of four BC_1F_1 plants having *T. repens* (CC-1) as the female parent and 8*x* H-435 as a male parent were also studied for somatic chromosome number and were confirmed to be hexaploid with $2n = 6x = 48$ (Fig. 2). All these BC_1F_1 plants theoretically combine four genomes from *T. repens* and two genomes from *T. ambiguum*.

The somatic chromosome numbers for four out of the seven BC_2F_1 (BC_1F_1 × CC-1) plants having BC_1F_1 as the female parent and *T. repens* (CC-1) as the male parent were studied and found to be pentaploid ($2n = 5x = 40$). These pentaploids are expected to carry four genomes from *T. repens* and one genome from *T. ambiguum*. The two plants obtained from the BC_1F_1 × 4*x T. ambiguum* (cv Treeline) cross were also found to be pentaploid ($2n = 5x = 40$, Fig. 2) but with an expectedly different genomic combination of two genomes derived from *T. repens* and three genomes from *T. ambiguum*.

Only two BC_1F_2 ($BC_1F_1 \times BC_1F_1$) plants were evaluated for somatic chromosome count and both were found to be hexaploids with $2n = 6x = 48$ (Fig. 2). One of these confirmed hexaploids was later used in crosses with 6*x T. ambiguum* (cv Prairie).

One of the two 4*x* H-435 × 8*x* H-435 plants studied for somatic chromosome count showed more than the expected 48 chromosomes (Fig. 2), the number being very close to 64. However, the chromosome number of this plant has not yet been confirmed. It might be an octoploid with $2n = 64$ or an aneuploid with definitely more than 48 chromosomes.

Pollen stainability

Pollen stainability data for all the material used in backcrosses, BC_1F_1, BC_2F_1, the congruity backcross and BC_1F_2 are presented in Table 2. Pollen stainabilities of six BC_1F_1 plants where 8*x* H-435 was used as the female parent averaged 37.9% (range 21.8–64.9%). Three reciprocal BC_1F_1 plants where *T. repens* (CC-1) was used as the female parent and 8*x* H-435 as the male parent gave an average pollen stainability of 22.8% (range 19.1–26.9%).

Average pollen stainability for the four pentaploid second backcross (BC_2F_1) derived using *T. repens* as the male parent was 59.3%, with a low of 44.4% and a high of 70.1%. One of the two 5*x* BC_2F_1 plants having 4*x T. ambiguum* (cv Treeline) as the male parent showed 17.6% stainable pollen. The other plant did not flower. Pollen stainability of the six BC_1F_2 plants was

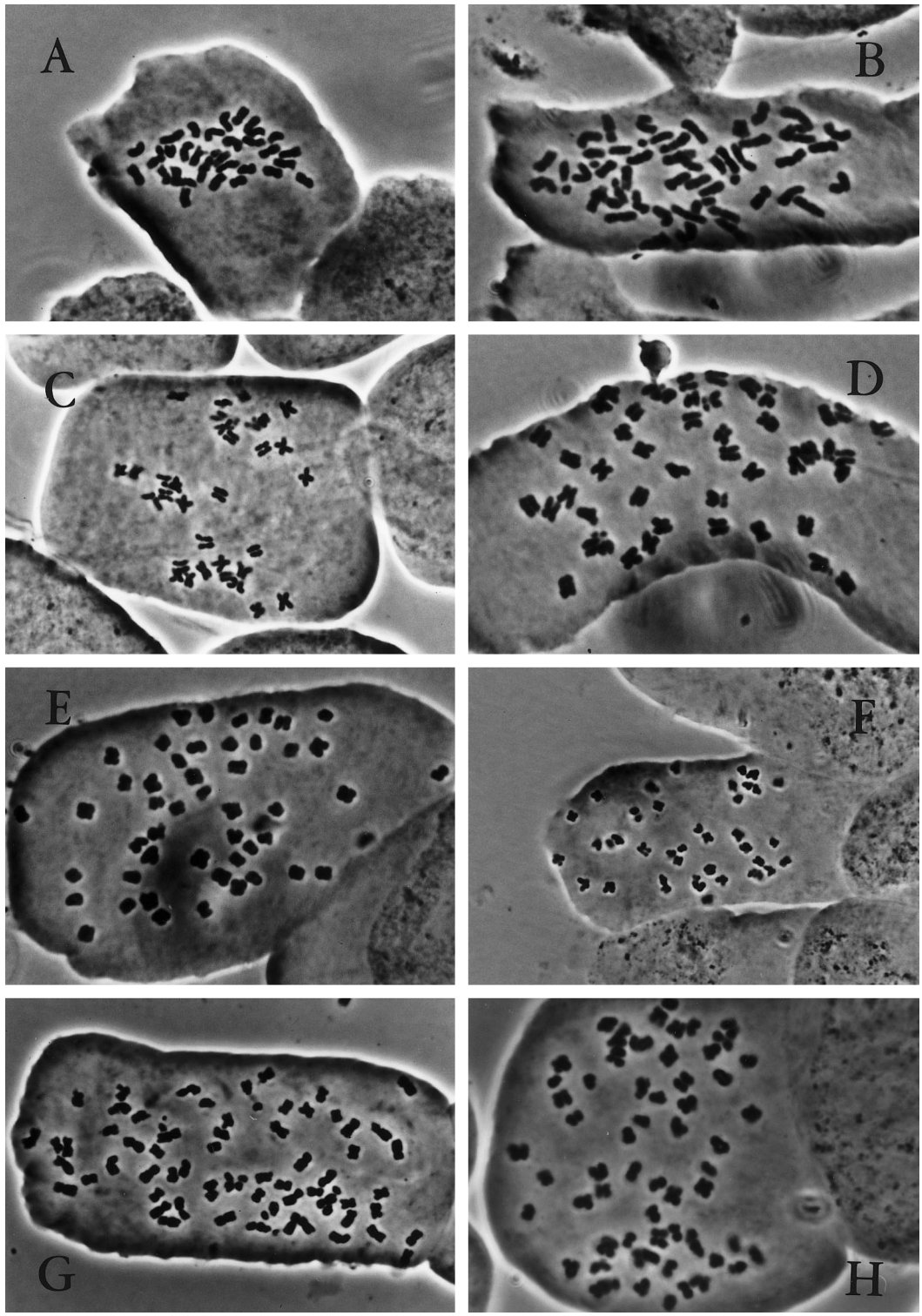

Fig. 2 Somatic chromosome numbers of (**A**) 4*x* *T. ambiguum* ($2n = 4x = 32$), (**B**) 6*x* *T. ambiguum* ($2n = 6x = 48$), (**C**) *T. repens* ($2n = 4x = 32$), (**D**) 6*x* BC_1F_1 (8*x* H-435 × *T. repens*, $2n = 6x = 48$), (**E**) 6*x* $BC_1F_1 \times 6x$ BC_1F_1 intercross (6*x* BC_1F_2, $2n = 6x = 48$), (**F**) 5*x* CBC_2 (6*x* $BC_1F_1 \times 4x$ *T. ambiguum*, $2n = 5x = 40$), (**G**) 8*x* H-435 ($2n = 8x = 64$) and (**H**) 4*x* H-435 × 8*x* H-435 ($2n$ = very close to 64). **A**, **C**, G = × 1600, **B**, **D** = × 1800, **E**, **H** = × 1650, **F** = × 1100

highly variable, ranging from about 1.0% to a high of 74.3%. The average was 40.8%.

Three CBC_2 progeny plants resulting from crosses between BC_1F_2 and 6*x* *T. ambiguum* showed pollen stainability averaging 93.6%, i.e. with a restoration of parental fertility levels.

Table 3 Somatic chromosome numbers, meiotic configurations of pollen mother cells (PMCs) and pollen stainability of *T. ambiguum* (cv Treeline), *T. repens* and first backcrosses of 8*x* H-435 to *T. repens*

Genotype/cross	Somatic chromosome no.	Total PMCs scored	Meiotic configuration at metaphase-I in pollen mother cells								Pollen stainability (%)
			I		II		III		IV		
			Mean	Range	Mean	Range	Mean	Range	Mean	Range	
T. ambiguum (cv Treeline)	$2n = 4x = 32$	18	0.39	(0–2)	15.39	(14–16)	0.05	(0–1)	0.17	(0–1)	96.8
T. repens (CC-1)	$2n = 4x = 32$	15	0.00	(0–0)	16.00	(16–16)	0.00	(0–0)	0.00	(0–0)	90.5
8*x* H-435 × (CC-1)-2	$2n = 6x = 48$	26	1.88	(0–3)	17.40	(14–21)	1.48	(0–3)	1.72	(0–3)	46.2
8*x* H-435 × (CC-1)-4	$2n = 6x = 48$	28	1.40	(0–3)	17.14	(12–21)	1.00	(0–2)	2.33	(0–4)	34.1
8*x* H-435 × (CC-1)-5	$2n = 6x = 48$	25	1.62	(0–4)	20.12	(16–22)	0.50	(0–2)	1.16	(0–2)	28.4
CC-1 × 8*x* H-435 × (CC-1)-2	$2n = 6x = 48$	27	3.13	(1–36)	17.70	(12–21)	1.45	(0–3)	1.28	(0–4)	22.5

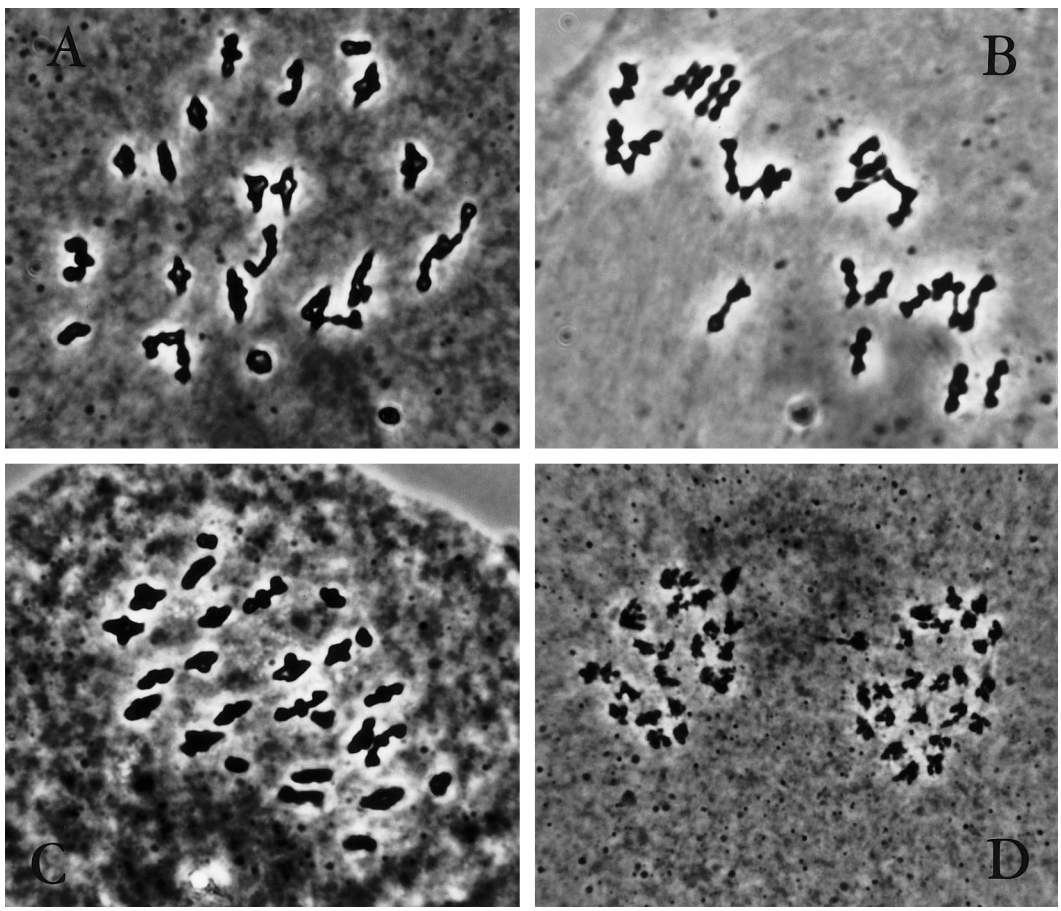

Fig. 3A–D Meiotic configuration in 6*x* BC_1F_1 (8*x* H-435 × *T. repens*) progeny. (**A**) 1·I + 16·II + 1·III + 3·IV at metaphase-I, (**B**) 20·II + 2·IV at metaphase-I, 4·I + 22·II at metaphase-I, and (**D**) 6*x* BC_1F_1 showing 24-24 disjunction at anaphase-I, × 1600

Meiotic configurations of 6*x* BC_1F_1 progeny

Results of meiotic chromosome pairing in pollen mother cells (PMCs) at metaphase-I for one genotype of 4*x* *T. ambiguum* (cv Treeline), *T. repens* (CC-1), and hexaploid BC_1F_1 are presented in Table 3. Meiotic chromosome pairings in 4*x* H-435 and 8*x* H-435 were not examined in the present study. Anderson et al. (1991) reported an average of 1.19 and 2.64 univalents, 14.34 and 27.62 bivalents, 0.25 and 0.74 trivalents and 0.34 and 0.84 quadrivalents for 4*x* and 8*x* H-435 respectively. The observation of 15 PMCs at metaphase-I in *T. repens* (CC-1) revealed only 16 bivalents per PMC, suggesting that meiosis is highly regular in this species. On the other hand, meiosis in one of the *T. ambiguum* (cv Treeline) plants was irregular in comparison to *T. repens*, forming on average 0.39 univalents,

0.06 trivalents, and 0.17 quadrivalents per PMC. However, 11PMCs showed normal 16-16 chromosome separation at anaphase-I, indicating that meiosis proceeded normally at the later stages.

Meiotic configurations were examined for 3 out of 15 hexaploid BC_1F_1 having 8*x* H-435 as the female parent and one out of four hexaploid BC_1F_1 having *T. repens* (CC-1) as the female parent. Pairing was found to be essentially similar in the four BC_1F_1 plants, with frequent univalent and multivalent formation (Table 3, Fig. 3). However, from the meiotic configurations of these four BC_1F_1 plants, it was impossible to distinguish the chromosomes of *T. repens* and *T. ambiguum* morphologically. Therefore, it was not possible from these observations to determine whether the bivalent and multivalent pairing was intra- or inter-specific. At least four PMCs in each of the BC_1F_1 plants observed at anaphase-I showed 24-24 chromosome disjunction (Fig. 3).

Discussion

First backcross (BC_1F_1)

Backcrosses of 4*x* H-435 to *T. repens* and 4*x* and 6*x* *T. ambiguum* were not successful. These results are partially consistent with the results of Williams and Verry (1981) and Anderson et al. (1991) who successfully obtained BC_1F_1 progeny of 4*x* H-435 to *T. repens* but failed to produce backcross progeny with *T. ambiguum.* The failure to obtain backcross progeny of 4*x* H-435 to *T. repens* in the present study might be due to the use of only one genotype of *T. repens* (CC-1) in backcrosses. Evans (1962) also observed that certain genotypes of one species showed greater compatibility than others in interspecific *Trifolium* crosses, suggesting that the growth and development of hybrid embryos may vary depending on the genotypes of the individual plants or strains that are crossed. Hovin (1962) found that *T. repens* used as a tester was more cross compatible with Italian than Turkish *T. nigrescens.* Thus backcrosses of 4*x* H-435 to *T. repens* reported by Williams and Verry (1981) and Anderson et al. (1991) may have been successful because they used different genotypes of white clover.

Self-compatibility of 4*x* H-435 has been reported by Williams and Verry (1981) and of 8*x* H-435 by Anderson et al. (1991). The production of 18 seeds from six selfed inflorescenes of 8*x* H-435 confirmed its self-compatibility. The failure of 4*x* H-435 to produce F_2 progeny in the present investigation might have been due to very poor dehiscence of the anthers with a very low frequency of viable pollen.

First-backcross progeny were successfully raised from reciprocal backcrosses of 8*x* H-435 to *T. repens.* However, backcrosses of 8*x* H-435 to 4*x* and 6*x* *T. ambiguum* were not successful. These results are again partially consistent with those of Anderson et al. (1991). These authors reported that 8*x* H-435 was both male- and female-fertile with *T. repens*, but was only female-fertile with 4*x* *T. ambiguum*, and produced 11 seeds from 528 backcrosses using 8*x* H-435 as the female parent. These seeds might have resulted from self-pollinations as their backcross origin was not confirmed.

The success of backcrossing 8*x* H-435 to *T. repens*, on the one hand, and its failure with 4*x* and 6*x* *T. ambiguum*, on the other hand, suggests that the relative dosages of genetic materials of the two species may regulate the development of backcrossed embryos (Rabakoarihanta et al. 1980). Somatic chromosomes were counted for eight BC_1F_1 plants having 8*x* H-435 as the female parent and *T. repens* as the male parent, and three reciprocal BC_1F_1 plants out of a total of 19 initially grown BC_1F_1 seeds. All were found to be hexaploid ($2n = 6x = 48$). This suggested that 8*x* H-435 was producing normal euploid ($n = 4x = 32$) gametes, thus giving a genomic combination of four *T. repens* and two *T. ambiguum* genomes in each 6*x* BC_1F_1.

Congruity (CBC) and second (BC_2F_1) backcrosses

The aim of generating 6*x* BC_1F_1 progeny between 8*x* H-435 and *T. repens* was to develop useful genetic material either for direct use as forage without the needs for further backcrossing to either parental species, or for inclusion in congruity backcrosses (Haghighi and Ascher 1988) to hexaploid *T. ambiguum* types that are considered to be agronomically superior to tetraploid types (Kannenberg and Elliot 1962; Spencer and Hely 1982). However, again, the congruity backcrosses of 6*x* BC_1F_1 to 6*x* *T. ambiguum* were not successful. Instead three seeds were harvested from one of the 6*x* BC_1F_1 plants pollinated with 4*x* *T. ambiguum* (cv Treeline). On the other hand, second backcrosses involving 6*x* BC_1F_1 and *T. repens* were more successful (Table 1). The failure of 6*x* BC_1F_1 to produce seeds in congruity backcrosses with 6*x* *T. ambiguum* and its success in second backcrosses with *T. repens* can once again be explained on the basis of the assumption that the relative dosages of the genetic material from the two parental species might seem to be operating in the development of backcross embryos. It is evident from the first (BC_1F_1) and second (BC_2F_1) backcrosses that those crosses were successful where both the female and male gametes presumably had the two homoeologous genomes of *T. repens.* Any deviation from this resulted in the failure of the cross. This suggests that in a hybrid environment the specific dosage of genetic material of one species (in this case *T. repens*) might have more influence on the regulation of the development of the backcrossed embryos than the other species. This was evident when 6*x* BC_1F_1 was reciprocally crossed with

6*x* *T. ambiguum*. Although the cross was made at the same (6*x*-6*x*) ploidy level the presence of only one member of each homoeologous pair from *T. repens* might be inadequate for normal development of the embryo in these backcrosses. On the other hand in backcrosses of 6*x* BC_1F_1 to *T. repens*, the gametes of 6*x* BC_1F_1 presumably had two homoeologous genomes of *T. repens*, resulting in the success of the second backcross.

Three out of seven initially grown BC_2F_1 (6*x* $BC_1F_1 \times T.$ *repens*) plants were evaluated for ploidy level and were found to be pentaploid ($2n = 5x = 40$) with presumably four genomes of *T. repens* and one genome of *T. ambiguum*. The two successfully grown 6*x* $BC_1F_1 \times 4x$ *T. ambiguum* (CBC_2) seeds were also pentaploid ($2n = 5x = 40$) but presumably with a different genomic combination, i.e. three genomes of *T. ambiguum* and two genomes of *T. repens*. The pentaploid BC_2F_1 plants with presumably four complete genomes of *T. repens* showed normal growth and development with an average of 59.3% pollen stainability (range 44.4–70.1%). In contrast, the two pentaploid CBC_2 plants exhibited developmental abnormalities. These abnormalities were expressed as altered leaf and inflorescence shape, low fertility, and very short internode length. Both plants reached flowering but one of them failed to produce normal flower heads and the inflorescences did not grow beyond the bud stage.

Developmental abnormality or hybrid breakdown, also referred to as hybrid sterility or weakness, arises in interspecific F_1 hybrids or hybrid derivatives as a result of lack of proper coordinated function of the genetic material contributed by the parents (Haghighi and Ascher 1988). These authors attributed the deviant growth and development of *Phaseolus* interspecific hybrids to incongruity, which has been defined as a pre- and/or post-zygotic reproductive barrier which results in the failure of intimate partner relationships because of a lack of genetic information in one partner about the critical factors of the other partner (Hogenboom 1973, 1984; Haghighi and Ascher 1988). Incongruity in a hybrid may therefore involve the mismatching of heritable information leading to the lack of coordinated development.The method of congruity backcrossing (defined as recurrent backcrossing of BC_1F_1 to each parent in alternate generations) has been suggested by Haghighi and Ascher (1988) to overcome incongruity barriers. Congruity backcrossing in *Phaseolus* (Haghighi and Ascher 1988) also produced individuals that exhibited symptoms of developmental incongruity (hybrid breakdown). Congruity backcrossing initially gives apparently slow improvement in fertility compared with recurrent backcrossing which can give rapid recovery in fertility but results in the loss of traits from the non-recurrent parent. However by the fourth or fifth congruity backcross generation, recombination seems to give rise to both recovered fertility and new and unique genetic combinations. The abnormal development and low fertility of the two pentaploid CBC_2 plants in the present investigation are consistent with the concept of incongruity.

$BC_1F_1 \times BC_1F_1$ intercrosses (BC_1F_2) and their use in congruity backcrosses

Anderson et al. (1991) suggested the possibility of obtaining (6*x* BC_1F_1 by crossing 8*x* H-435 to 4*x* *T. ambiguum*. These hexaploid BC_1F_1 would be expected to carry four genomes of *T. ambiguum* and two genomes of *T. repens*. In fact, they were able to produce 11 seeds from 528 backcrosses using 8*x* H-435 as the female parent but, as already mentioned, these authors did not evaluate the plants for backcross origin. In the present investigation the backcrosses of 8*x* H-435 to 4*x* *T. ambiguum* failed and so another approach was adopted to generate similar hexaploids. This involved the crossing of already existing 6*x* BC_1F_1 (with presumably four genomes of *T. repens* and two genomes of *T. ambiguum*) with 6*x* *T. ambiguum* in congruity backcrosses. However, the cross failed once again despite the same 6*x*-6*x* ploidy level of the parents. Alternatively, the 6*x* BC_1F_1s were intercrossed among each other with the idea of selecting a more-fertile 6*x* BC_1F_2 and testing its effectiveness in crosses with 6*x* *T. ambiguum*. At this stage one, [(CC-1 × 8*x* H-435)-2 × (CC-1 × 8*x* H-435)-2]-1, out of six 6*x* BC_1F_2 plants produced seeds when pollinated with 6*x* *T. ambiguum* (Table 1).

Assuming that only a small amount of allosyndetic pairing among the genomes of 6*x* BC_1F_1 was occurring, the resulting 6*x* BC_1F_2 progeny from 6*x* $BC_1F_1 \times 6x$ BC_1F_1 intercrosses would presumably have an altered genetic (not necessarily genomic) combination as a result of recombination. Perhaps this altered genetic combination in one of the 6*x* BC_1F_2 plants has resulted in its cross compatibility with 6*x* *T. ambiguum*. Three plants from 6*x* $BC_1F_2 \times 6x$ *T. ambiguum* have been grown in the glasshouse. These plants have pollen stainabilities averaging close to the parental fertility of around 95%, and represent the culmination of a progression (Table 2) from 13.3% in the 4*x* primary hybrid through 50–70% in some BC_1F_2 plants to the current very high level. These plants will be evaluated cytologically for ploidy level and meiotic chromosome configurations at later stages. Thus the sequence of crosses shown in Table 2 has provided useful genetic material for transferring characters of interest from both parental species and can be used as a "bridge" between *T. repens* and *T. ambiguum*. Anderson et al. (1991) had earlier suggested that the use of crosses between *T. repens* and the octoploid hybrid (8*x* H-435) may have limited value because of a depleting number of *T. ambiguum* chromosomes. The new approach described in the present investigation overcomes this difficulty.

The present investigation has resolved the difficulties originally perceived for the combining of superior white clover and Caucasian clover traits without having to resort to further primary hybrids between these species. The solution is to first hybridise superior white clover plants with 8*x* H-435 to generate 6*x* progenies selectable for superior white clover traits. Second, these selected 6*x* plants are recombined (intercrossed) to give 6*x* plants which will hybridise with 6*x* *T. ambiguum*. Third, superior 6*x* *T. ambiguum* plants are crossed with these selected 6*x* hybrids (white clover backcrosses) as a "fertile bridge" to effectively combine superior traits from both species. Further intercrosses among the 6*x* progenies should produce new combinations and genomic balances yet to be researched.

Meiotic configurations of 6*x* BC_1F_1

The ultimate outcome of interspecific hybridisation will depend to a considerable extent on the degree of pairing and genetic exchange between chromosomes of the two species. The average frequency of 1.1 trivalents and 1.6 quadrivalents in four 6*x* BC_1F_1 plants in the present experiment is higher than the earlier findings of Anderson et al. (1991) who reported an average of 0.5 trivalents and 0.07 quadrivalents in two 6*x* BC_1F_1 plants. They also found predominantly bivalent pairing, i.e. an average of 22.5 bivalents in contrast to an average of 18.0 bivalents in the present study. Although presumably possessing the same genomic combination of the two parental species (four genomes of *T. repens* and two genomes of *T. ambiguum*), the origin of the 6*x* BC_1F_1s generated in the present investigation is different from those reported by Anderson et al. (1991) where the 6*x* BC_1F_1 was obtained through unreduced gametes contributed by the 4*x* H-435 female parent. Whether this difference in meiotic configurations was due to the difference in the backcross origin of the two sets of 6*x* BC_1F_1s is uncertain.

Chromosomes of *T. repens* and *T. ambiguum* were not distinguishable in meiotic cells of 6*x* BC_1F_1 plants. Therefore, it was not possible to conclude whether the bivalent and multivalent pairing in these plants represents intra- or inter-specific chromosome pairing. Meiotic configurations for the 4*x* H-435 were reported to be mostly bivalent with a low frequency of multivalent formation (Williams et al. 1982; Anderson et al. 1991). It was impossible for these authors to conclude whether predominantly bivalent formation represented the pairing of intraspecific homoeologous chromosomes (autosyndesis) or pairing between *T. repens* and *T. ambiguum* chromosomes (allosyndesis). However, the multivalent formation can only have been the result of both types of pairing.

Results obtained by Williams et al. (1982) for the meiotic configurations of the single tetraploid BC_1F_1 plant from backcrosses of 4*x* H-435 to *T. repens* (with presumably three genomes of *T. repens* and one genome of *T. ambiguum*) exhibited extreme meiotic irregularities with an average of ten univalents per pollen mother cell. The multivalent (an average of 3.15 trivalents and 0.13 quadrivalents) formation in that 4*x* BC_1F_1 plant was suspected to be the result of homoeologous pairing of *T. repens* or *T. ambiguum* chromosomes with a homologous *T. repens* pair. In contrast, Anderson et al. (1991) observed mostly bivalent (an average of 15.71 per PMC) pairing in two similar backcross plants. The reasons for these contrasting results are not known and Anderson et al. (1991) have not attempted to provide a possible explanation for the differences in meiotic configurations of their two 4*x* BC_1F_1 plants and that of Williams et al. (1982). The observations of Anderson et al. (1991), however, provided firm support for a large amount of allosyndetic pairing. In the 32 chromosome progeny of the first backcrosses of 4*x* H-435 to *T. repens*, one homologous set of *T. repens* chromosomes was believed to be pairing as eight bivalents, with the remaining eight *T. repens* chromosomes pairing as bivalents with *T. ambiguum* chromosomes.

Based on these assumptions it can be further assumed that, in the presence of homologous genomes of white clover, homoeologous white clover chromosome pairing (autosyndesis) has been largely suppressed, thus allowing the third genome of white clover to pair mainly allosyndetically with Caucasian clover chromosomes in the 32-chromosome BC_1F_1 plants. With regard to the 6*x* BC_1F_1 plants obtained in the present investigation, it can be assumed that four genomes of *T. repens* might pair mainly as homologues, with the two genomes of *T. ambiguum* pairing as homoeologues. Multivalent formation might be the result of the autosyndetic pairing of homoeologous *T. repens* chromosomes or the allosyndetic pairing of *T. repens* and *T. ambiguum* chromosomes. This assumption is consistent with the earlier findings of Anderson et al. (1991).

Based on the results obtained for meiotic configurations of 6*x* BC_1F_1 plants in the present investigation, and the results of Anderson et al. (1991) for 4*x* H-435, 4*x* BC_1F_1 and 6*x* BC_1F_1 plants, it can be assumed that, if the backcross progeny have an odd number of *T. repens* genomes, there will be a greater frequency of allosyndetic pairing, i.e. the odd genome of *T. repens*, having no homologous set of chromosomes to pair with, might provide greater chances for allosyndetic pairing.

Apart from the 6*x* BC_1F_1 plants, a pentaploid second-backcross progeny (5*x* BC_2F_1) with presumably four genomes of *T. repens* and one genome of *T. ambiguum* was also produced in the present investigation. Although these 5*x* BC_2F_1 plants were not studied for meiotic configurations, they are likely to be potential candidates for further studying the chromosomal relations of these two species.

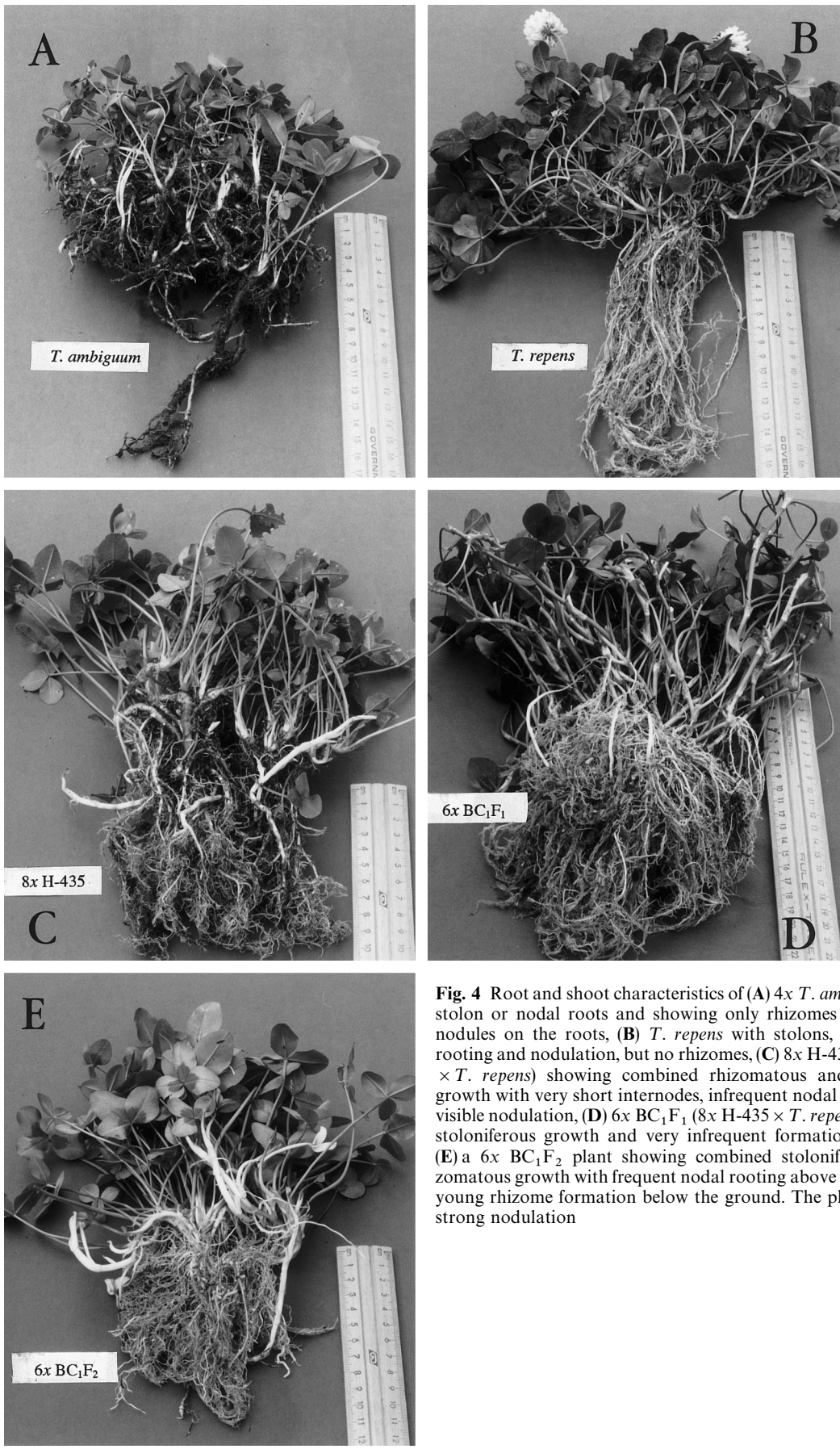

Fig. 4 Root and shoot characteristics of (**A**) 4*x* *T. ambiguum* with no stolon or nodal roots and showing only rhizomes and no visible nodules on the roots, (**B**) *T. repens* with stolons, frequent nodal rooting and nodulation, but no rhizomes, (**C**) 8*x* H-435 *T. ambiguum* × *T. repens*) showing combined rhizomatous and stoloniferous growth with very short internodes, infrequent nodal rooting and no visible nodulation, (**D**) 6*x* BC_1F_1 (8*x* H-435 × *T. repens*) with mostly stoloniferous growth and very infrequent formation of rhizomes, (**E**) a 6*x* BC_1F_2 plant showing combined stoloniferous and rhizomatous growth with frequent nodal rooting above the ground and young rhizome formation below the ground. The plant also shows strong nodulation

Conclusions

Beginning with an almost-sterile tetraploid hybrid plant and a derived octoploid that was only a little more fertile, a range of fertile hybrids now exists at 5*x*, 6*x* and 8*x* levels with varying balances of chromosome sets from the parental species. The creation of these backcross populations at different ploidy levels will enable the development of new clover types which are, for example, mostly like white clover but carry some attributes (like the root system) from Caucasian clover. Already some of the 6*x* backcross plants are showing combined stoloniferous and rhizomatous growth (Fig. 4) which may provide a vigorous but highly persistent legume for sustainable systems in difficult environments.

Although taxonomically related, *T. ambiguum* and *T. repens* are very difficult to cross. The rare hybrids so far reported between these two species were obtained only by embryo culture. This difficulty has been overcome in the present research by the creation of a "fertile bridge" between *T. ambiguum* and *T. repens*. In effect starting with a single 8*x* hybrid plant of low fertility, the way has now been opened for the breeding of new types of clovers. Characters of interest can now be transferred from *T. ambiguum* to *T. repens* by using the 6*x* BC_1F_2 plants as a "fertile bridge" without the use of sophisticated techniques like embryo culture with nurse endosperm or ovule culture.

Acknowledgements We thank Associate Prof. J. G. Hampton and Drs. D. R. Woodfield, E. G. Williams and N. L. Taylor for valuable discussions. S. W. Hussain was the recipient of a New Zealand Ministry of Foreign Affairs and Trade Fellowship. The provision of facilities by AgResearch Grasslands, Palmerston North is gratefully acknowledged.

References

Alconero R, Fiori B, Sherring W (1986) Relationships of virus infection to field performance of six clover species. Plant Dis 70:119–121

Anderson JA, Taylor NL, Williams EG (1991) Cytology and fertility of the interspecific hybrid *Trifolium ambiguum* × *T. repens* and backcross populations. Crop Sci 31:683–687

Barnett OW, Gibson PB (1975) Identification and prevalence of clover viruses and resistance of *Trifolium* species to these viruses. Crop Sci 15:32–37

Brewbaker JL, Carnahan HL (1956) Leaf marking alleles in white clover – uniform nomenclature. J Hered 47:103–104

Bryant WG (1974) Caucasian clover (*Trifolium ambiguum* Bieb.): a review. J Aust Inst Agric Sci 40:11–19

Carnahan JL, Hill HD, Hanson AA, Brown KG (1955) Inheritance and frequencies of leaf markings in white clover. J Hered 46:109–114

Evans AM (1962) Species hybridisation in *Trifolium*. II. Investigating the pre-fertilisation barriers to compatibility. Euphytica 11:256–262

Gaynor DL, Skipp RA (1987) Pests. In: Baker MJ, Williams WM (eds) White clover. CAB International, Wallingford, Oxon, pp 461–492

Giri N, Taylor NL, Collins GB (1981) Chromosome numbers in some *Trifolium* species with a karyotype of *T. physodes*. Can J Genet Cytol 23:621–626

Haghighi KR, Ascher PD (1988) Fertile, intermediate hybrids between *Phaseolus vulgaris* and *P. acutifolius* from congruity backcrossing. Sex Plant Reprod 1:51–58

Hogenboom NG (1973) A model for incongruity in intimate partner relationships. Euphytica 22:219–233

Hogenboom NG (1984) Incongruity: non-functioning of intercelluar and intracellular partner relationships through non-matching information. In: Linskens HF, Heslop-Harrison J (eds) Cellular interactions. Encyclopedia of Plant Physiology NS vol. 17, Springer-Verlag, Berlin, pp 641–654

Hovin AW (1962) Interspecific hybridization between *Trifolium repens* L. and *T. nigrescens* Viv. and analysis of hybrid meiosis. Crop Sci 2:251–254

Jones RA, Rupert EA, Barnett OW (1981) Virus infection of *Trifolium* species in cell suspension cultures. Phytopathology 71:116–119

Kannenberg LW, Elliott FC (1962) Ploidy in *Trifolium ambiguum*, M. Bieb. in relation to some morphological and physiological characters. Crop Sci 2:378–381

Lenoble M, Papineau J (1970) Note sur une nouvelle marque foliaire chez *Trifolium repens*. Ann Amelior Plant (Paris) 20:485–487

McLaughlin MR, Pederson GA (1985) Coincidence of virus disease and decline of white clover in a Mississippi pasture (abstract). Plant Dis 75:1359

Mercer CF (1988) Reaction of some species of *Trifolium* to *Meloidogyne hapla* and *Heterodera trifolii*. Proc 5th Aust Conf Grassland Invertebrate Ecology, Melbourne, pp 275–280

Meredith MR, Michaelson-Yeates TPT, Ougham HJ, Thomas H (1995) *Trifolium ambiguum* as a source of variation in the breeding of white clover. Euphytica 82:185–191

Pederson GA, McLaughlin MR (1989) Resistance to viruses in *Trifolium* interspecific hybrids related to white clover. Plant Dis 73:997–999

Pederson GA, Windham GL (1989) Resistance to *Meloidogyne incognita* in *Trifolium* interspecific hybrids and species related to white clover. Plant Dis 73:567–569

Rabakoarihanta A, Shii CT, Mok MC, Mok DWS (1980) Meiosis and fertility of interspecific hybrids between *Phaseolus vulgaris* L. and *P. acutifolius* A. Gray. Theor Appl Genet 57:59–64

Ragland CK, Campbell CL, Moyer JW (1986) The effect of clover yellow vein virus and peanut stunt virus on yield of two clones of ladino white clover. Phytopathology 76:557–561

Skipp RA, Gaynor DL (1987) Pest-nematodes. In: Baker MJ, Williams WM (eds) White clover. CAB. International, Wallingford, Oxon, pp 493–512

Snow R (1963) Alcoholic hydrochloric acid-carmine as a stain for chromosomes in squash preparations. Stain Technol 38:9–13

Spencer K, Hely FW (1982) Shoot and root responses to phosphorus by *Trifolium ambiguum* and *Trifolium repens* in a montane environment. NZ J Agric Res 25:77–82

Spencer K, Hely FW, Govaars AG, Zorin M, Hamilton LJ (1975) Adaptability of *Trifolium ambiguum* Bieb. to a Victorian montane environment. J. Aust Inst Agric Sci 41:268–270

Taylor NL, Anderson MK, Quesenberry KH, Watson L (1976) Doubling the chromosome number of *Trifolium* species using nitrous oxide. Crop Sci 16:516–518

Taylor NL, Anderson JA, Williams EG, Williams WM (1991) Registration of octoploid hybrid clover germplasm from the cross of *Trifolium ambiguum* × *T. repens*. Crop Sci 31:1395

Westbrook FE, Tesar MB (1955) Tap-root survival of ladino clover. Argon J 47:403–410

Williams EG (1978) A hybrid between *Trifolium repens* and *T. ambiguum* obtained with the aid of embryo culture. NZ J Bot 16:499–506

Williams EG, Verry IM (1981) A partially fertile hybrid between *Trifolium repens* and *T. ambiguum*. NZ J Bot 19:1–7

Williams EG, Plummer J, Phung M (1982) Cytology and fertility of *Trifolium repens*, *T. ambiguum*, *T. hybridum* and interspecific hybrids. NZ J Bot 20:115–120

Williams EG, Taylor NL, van den Bosch J, Williams WM (1990) Registration of tetraploid hybrid clover germ plasm from the cross of *Trifolium ambiguum* × *T. repens*. Crop Sci 30:427

Williams WM (1987) Genetics and breeding. In: Baker MJ, Williams WM (eds) White clover. CAB. International Wallingford, Oxon, pp 343–419

Williams WM (1954) An emasculation technique for certain species of *Trifolium*. Agron J 46:182–184

Yamada T, Fukuoka H (1986) Production of interspecific hybrids between *Trifolium ambiguum* M. Bieb. and *T. repens* L. by ovule culture. Jpn J Breed 36:233–239

Yamada T, Fukuoka H, Higuchi S (1989) Interspecific hybridization of tetraploid kura clover (*Trifolium ambiguum* M. Bieb.) and white clover (*T. repens* L.) using ovule culture. J Jpn Soc Grassland Sci 35:180–185

Yeates GW, Healy WB, Widdowson SP (1973) Screening of legume varieties for resistance to the root nematodes *Heterodera trifolii* and *Meloidogyne hapla*. NZ J Agric Res 16:81–86