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Widening the adaptation of white clover by incorporation of valuable new traits from wild clover species

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Abstract. Although white clover (*Trifolium repens*) is the most widely used legume in grazed pastures of temperate and sub-tropical regions, it is severely restricted in genetic diversity for adaptive traits to low soil fertility and other stress environments, including drought. The objective of this research was to transfer traits for wider adaptation from other clover species by hybridisation. Eight *Trifolium* species with contrasting adaptations were shown by DNA sequence phylogenetics to be closely related to white clover. Interspecific hybridisation was undertaken among these species using embryo rescue, and an array of partially fertile F₁ hybrids was obtained. Population development from these F₁ hybrids showed that hybrids involving six taxa could be selected for high sexual fertility. Most showed strong inter-species chromosome pairing and the potential for introgression of exotic genomes into white clover. Several of the new genomic combinations, which do not occur in nature, will improve prospects for extending the adaptive range of white clover.

Keywords: *Trifolium repens*, interspecific hybridisation, *Trifolium ambiguum*, *Trifolium occidentale*, *Trifolium nigrescens*.

Introduction

White clover is the pasture legume most used in grazed pastures in moist temperate regions. However, it is poorly adapted to semi-arid or dry, infertile soils and is susceptible to a wide array of pests. Searches for white clover populations with strong adaptations to such conditions have been unsuccessful. However, white clover and its progenitors and close relatives belong to a group that is undergoing adaptive radiation from a relatively recent common ancestor (Williams *et al.* 2012). This has considerable agricultural significance as natural selection in diverse environments has led to genomes with valuable traits for adaptation to stress environments. Consequently, there are several other *Trifolium* species that have been crossed with white clover that do have greater genetic variation for these and other traits. For example, *T. ambiguum* has been identified as tolerant to drought and several pests and crossed with white clover (Williams and Verry 1981, Meredith *et al.* 1985). Similarly, *T. nigrescens* has nematode resistance (Hussain *et al.* 1987) and profuse flowering (Marshall *et al.* 2008), both of which have been transferred to white clover in interspecific hybrids. Several other species have been crossed with white clover to give fertile progeny, including *T. uniflorum* (Gibson *et al.* 1971, Pandey *et al.* 1987), *T. occidentale* (Gibson and Beinhart 1969) and *T. isthmocarpum* (Ferguson *et al.* 1990).

Use of the secondary and tertiary gene pools for plant breeding involves four to five main steps: (1) identification of wild relatives with the required traits, (2) achievement of F₁ interspecific hybrids with the wild species, (3)

development of secondary hybrids between the primary hybrid and the target species, (4) achievement of large breeding populations of fertile interspecific hybrids and, (5) screening and selection of cultivars from these large populations. To-date, the large-scale breeding steps 4-5 have been reached on a limited scale only for *T. ambiguum* x *T. repens* and *T. repens* x *T. nigrescens* hybrids. The remainder have generally not advanced past the experimental hybrid (pre-breeding) stages (2-3).

We have previously reported our clover interspecific hybrid results up to the pre-breeding step 2 with white clover and the species listed in Table 1 (Williams *et al.* 2006). Here we report further developments in pre-breeding of clover interspecific hybrids as well as advancement to large breeding populations (step 4) of combinations involving white clover and *Trifolium* species with potentially valuable new trait combinations.

Materials and Methods

Plants of all species with a close phylogenetic relationship with white clover (Ellison *et al.* 2006) (Table 1) were derived as seeds from the Margot Forde Germplasm Centre, Palmerston North, New Zealand. For hybridisation all species were grown in pots in an insect-free greenhouse. Chromosome-doubled (4x) forms were produced by colchicine treatment of 2x *T. occidentale*, 2x *T. ambiguum*, 2x (*T. ambiguum* x *T. occidentale*) and 2x (*T. pallescens* x *T. occidentale*) (Williams *et al.* 2011). Emasculation and cross pollination methods followed the method of Williams and Verry (1981). Few of the hybrid combinations set

Table 1. *Trifolium* species, ploidy, genome designation, perennial or annual (P/A).

Species	Ploidy/Genome	P/A	Distribution	Habitat
<i>T. occidentale</i>	2x/O	P	W Europe	Coastal sand dunes
<i>T. pallescens</i>	2x/P	P	Europe	High alpine
<i>T. thalii</i>	2x/T	P	Europe, N Africa	Mid-high altitude grassland
<i>T. nigrescens</i>	2x/N	A	Mediterranean, W Asia	Open ground, shrubland
<i>T. isthmocarpum</i>	2x/I	A	N Africa, Mediterranean	Moist fields, hills
<i>T. uniflorum</i>	4x/U	P	Mediterranean	Fields, scrub, slopes
<i>T. montanum</i>	2x, 4x/M	P	Europe, Caucasus	Dry grassland-alpine
<i>T. ambiguum</i>	2x/A ^D , 4x/A ^T , 6x/A ^H	P	Caucasus	High altitude slopes, fields
<i>T. repens</i>	4x/R	P	Europe, W. Asia	Moist, temperate grassland, slopes

Table 2. F₁ hybrid combinations developed, numbers obtained, crossing method (N=natural, ER=embryo rescue), expected ploidy/genomic constitution, pollen stainability (%) and seed-set (infl = inflorescence).

	Number	Ploidy/genomes	Pollen stainability	Seed-set
4x <i>T. ambiguum</i> x <i>T. repens</i>	3 (ER)	4x, A ^T A ^T RR	2-9%	0.2-2.8/infl
<i>T. repens</i> x <i>T. uniflorum</i>	50-70 (ER)	4x, RRUU	0-90%	0-16/infl
<i>T. repens</i> x 4x <i>T. occidentale</i>	Many, N	4x, RROO	0-70%	1-43/100 florets
<i>T. repens</i> x <i>T. nigrescens</i>	Many, N	3x, RRN	0-16%	0-1.1/infl
<i>T. nigrescens</i> x <i>T. occidentale</i>	A few, N	2x, NO	57%	1-8/infl
<i>T. pallescens</i> x <i>T. occidentale</i>	4 (ER)	2x, PO	<1-5%	<1/infl
2x <i>T. ambiguum</i> x 2x <i>T. occidentale</i>	3 (ER)	2x, AO	2-3%	0.8 seeds/infl
4x <i>T. ambiguum</i> x 2x <i>T. occidentale</i>	1 (ER)	3x, AAO	9%	1/infl
4x <i>T. ambiguum</i> x 4x <i>T. occidentale</i>	5 (ER)	4x, AAOO	40-90%	0-55/infl
6x <i>T. ambiguum</i> x 2x <i>T. occidentale</i>	6 (ER)	4x, AAAO	1-5%	0-14/100 infl
6x <i>T. ambiguum</i> x 2x <i>T. pallescens</i>	2 (ER)	4x, AAAP	3-8%	<1/100 infl
6x <i>T. ambiguum</i> x 2x <i>T. thalii</i>	2 (ER)	4x, AAAT	8-25%	0-1/infl

Table 3. Secondary and tertiary hybrids setting seed by natural crossing (not requiring ER). Genomes in brackets show introgression. Interspecific chromosome pairing strong, +; weak (+); unknown, ND.

	Expected genomes	Chromosome pairing	Pollen stainability	Seed-set
2x, 4x (<i>T. pallescens</i> x <i>T. occidentale</i>) x <i>T. repens</i>	PORR	+	0-78%	2-41/infl
4x (<i>T. pallescens</i> x <i>T. occidentale</i>) selfed	PPOO	+	78%	4-19/infl
<i>T. repens</i> x 4x (<i>T. repens</i> x <i>T. uniflorum</i>)	RRRU and RRRR(U)	+	10-90%	0-150/infl
<i>T. repens</i> x 4x (<i>T. repens</i> x <i>T. occidentale</i>)	RRRO and RRRR(O)	+	23-95% 30-87%	15-200/infl 7-200/infl
3x (<i>T. repens</i> x <i>T. nigrescens</i>) x <i>T. repens</i>	RRRR(N)	+	15-90%	20-150/infl
4x (<i>T. ambiguum</i> x <i>T. repens</i>) x <i>T. repens</i>	A ^T A ^T RRRR, A ^D A ^T RRRR	ND	37-98% 84-95%	30-140/infl 75/infl
4x (<i>T. ambiguum</i> x <i>T. occidentale</i>)	AAOO	(+)	0-98%	0-120/infl

natural seed (Table 2) and embryo rescue (ER), as described in Williams *et al.* (2011), was used to achieve most successful crosses.

Mature plantlets were transferred from tissue culture to potting mix and putative hybrids were grown to maturity in a greenhouse. On maturity they were verified by DNA sequence, isozyme and molecular cytogenetic analyses (Williams *et al.* 2011). Female fertility was assessed from seed-set, male fertility from pollen stainability and chromosome pairing by analysis of meiotic chromosome configurations in pollen mother cells (Williams *et al.* 2011). For the subsequent generations, seedlings were grown and treated in the same way as the explants.

Results

The species of the white clover complex are from diverse

habitats (Table 1) and potentially carry traits for drought, cold, salt and pest resistances, as well as possible adaptation to low soil fertility. Virtually all of these traits are likely to be conditioned by alleles not currently available in the white clover gene pool. The fertile or partially fertile F₁ hybrids developed from the crossing programme are listed in Table 2. Seven of the more fertile hybrid combinations had sufficient fertility to enable generation of progeny by natural crossing (Table 3).

All of these combinations had adequate fertility to be advanced to large breeding populations for cultivar selection (steps 4 and 5). Four further F₁ combinations had retained low fertility (Table 4) and could only be advanced by ER. Three have continued to show low fertility, and remain as potentially valuable pre-breeding populations that require further improvement (Table 4). The fourth

Table 4. Secondary or tertiary hybrids derived by crossing infertile hybrids using ER.

Female x male	Genomes	Pollen stainability	Seed-set
2x <i>T. ambiguum</i> x 6x A ^T A ^T RRRR	A ^D A ^T RR	6-55%	0.5-3/infl
<i>T. repens</i> x 4x (<i>T. ambiguum</i> x <i>T. occidentale</i>)	RRA ^T O RRA ^D O	Most <10%	0-2/infl
6x (<i>T. ambiguum</i> x 2x <i>T. occidentale</i>) x <i>T. repens</i>	A ^H A ^H A ^H RRO	2-60%	0-10/infl
6x <i>T. ambiguum</i> x 6x A ^T A ^T RRRR	A ^H A ^H A ^H A ^T RR	0-18%	<1/infl

(A^DA^TRR) was developed by ER, and was then fertile enough to enable development of 6x breeding populations by natural crossing

Discussion

Populations large enough to move from the small-scale pre-breeding phase to full-scale plant breeding with thousands of plants were achieved for hybrid combinations of white clover with five other species (six taxa). These included four 4x hybrid combinations in which white clover was potentially introgressed by *T. uniflorum*, *T. occidentale*, *T. pallescens* and *T. nigrescens* and two 6x combinations of *T. repens* with 2x and 4x *T. ambiguum*. In addition, the 4x combination of *T. occidentale* with either or both of 2x and 4x *T. ambiguum* represented a potential new species that had not occurred in nature (because of geographic separation) and had not been created artificially (because of the difficult hybridisation). In each case, the successful development of breeding populations had overcome one or more post-fertilisation barriers, including low hybrid fertility, endosperm failure (ER), and unbalanced ploidal levels. The ability of these wide hybrids to express traits from the donor species has been well demonstrated by the confirmed drought tolerances of *T. repens* x *T. ambiguum* (Marshall *et al.* 2001) and *T. repens* x *T. uniflorum* hybrids (Nichols *et al.* this conference) and the expressions of nematode resistance (Hussain *et al.* 1987) and profuse flowering in *T. repens* x *T. nigrescens* hybrids (Marshall *et al.* 2008). One of the most potentially desirable taxa to integrate with the white clover genome, 6x *T. ambiguum*, has not yet joined this list. However, there were at least two promising combinations, still at the pre-breeding stage (Table 4).

Because of the recent radiation of species in the white clover complex, the genomes remain similar enough to permit hybridisation and, in some cases, introgression. Although *T. ambiguum* formed hybrids with several species, limited results obtained to-date suggested that *T. ambiguum* chromosomes showed interspecific pairing only at low frequencies. An exception was the high pairing of *T. ambiguum* and *T. occidentale* chromosomes in an AO hybrid (Williams *et al.*, 2011), leading to the subsequent use of *T. occidentale* as a genetic bridge, as in Table 4.

Conclusion

Interspecific hybridisation enables the transfer of valuable new traits to extend the adaptive range of white clover into more marginal environments. The development of new hybrid combinations will further improve prospects for extending the adaptive range of future white clover cultivars.

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