Theor Appl Genet (2001) 103:1211–1215

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Genetic polymorphism for cyanogenesis and linkage at the linamarase locus in *Trifolium nigrescens* Viv. subsp. *nigrescens*

Received: 27 December 2000 / Accepted: 12 April 2001

Abstract Polymorphisms at two genetic loci conditioning the cyanogenic glucoside linamarin (*Ac*) and the glucosidase linamarase (*Li*) are reported for the first time in *Trifolium nigrescens* Viv. subspecies *nigrescens* (2*n*=2*x*=16). *T. nigrescens* is one of several possible ancestral species that may have donated a genome to the allotetraploid species white clover (*T. repens* L., 2*n*=4*x*=32). *T. nigrescens* is a strong candidate because it is the only very close relative that, like white clover, is cyanogenic. Genetic analysis showed that in *T. nigrescens*, cyanogenesis was inherited as a two-locus genetic system in a similar way to that in white clover. Furthermore, *Li*, which is linked to the locus *Sdh* (shikimate dehydrogenase, SDH) at a distance of 6 cM in one genome of white clover, also showed linkage (12 cM) in *T. nigrescens*. It is concluded that one of the subspecies of *T. nigrescens* is a likely donor of a genome to white clover.

Keywords *Trifolium nigrescens* · *Trifolium repens* · Cyanogenesis · Linamarase · Shikimate dehydrogenase

Introduction

Trifolium nigrescens Viv. is an annual clover species distributed throughout the Mediterranean region and Asia Minor, and inland to the Caucasus. Three distinct subspecific taxa (two subspecies and one variety) have been identified (Hossain 1961; Williams et al. 2000). Subspecies *nigrescens* is distributed naturally throughout the Mediterranean countries of Europe, western Turkey, Crete and North Africa, while the other two subspecies have a more eastern occurrence in Turkey and the Caucasus (Hossain 1961; Zohary and Heller 1984).

Communicated by G. Wenzel

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T. nigrescens is a putative donor of one genome to the important tetraploid agricultural species, white clover, *Trifolium repens* L. which is a probable amphidiploid (Williams 1987). Evidence that *T. nigrescens* is a present-day ancestor of *T. repens* includes chromosome pairing in artificial *T. repens*×*T. nigrescens* hybrids (Brewbaker and Keim 1953; Chen and Gibson 1970; Hovin 1962; Kakes and Hakvoort 1994; Hussain et al. 1997) and chromosomal locations of rDNA loci (Ansari et al. 1999; Williams et al. 2000). *T. repens* carries the genetic loci for the cyanogenic glucosides linamarin and lotaustralin (*Ac*), and the hydrolysing enzyme linamarase (*Li*), in disomic rather than tetrasomic form, presumably having received these simultaneously from one ancestral parent and not the other. The only diploid species which are closely related to *T. repens* and are cyanogenic (and therefore presumably carry *Ac* and *Li*) are *T. nigrescens* and *Trifolium montanum*. Of these, *T. nigrescens*, is the more-likely candidate, based on other evidence, and comparison of linamarase structures shows a general resemblance between the linamarases of the two species (Kakes and Hakvoort 1994), further strengthening this hypothesis.

T. nigrescens had previously been found to be monomorphic for cyanogenesis and so it had not been possible to compare the genetics of this trait in the two species. In this paper we report, for the first time, polymorphism at both the *Ac* and *Li* loci in *T. nigrescens*. We then use this polymorphism to test the hypothesis that the two-locus genetic model of cyanogenesis in *Trifolium repens* also applies to *T. nigrescens*. Furthermore, in *T. repens* the linamarase locus (*Li*) and the shikimate dehydrogenase locus (*Sdh*) had previously been shown to be closely linked (Williams et al. 1998). Here we test the hypothesis that if *T. nigrescens* was the donor of the cyanogenic trait to *T. repens*, the two species would share this linkage.

Materials and methods

Eighteen accessions of *T. nigrescens* identified as subspecies *nigrescens* (Williams et al. 2000) were obtained from the Margot Forde Germplasm Centre, Palmerston North, New Zealand. All

were screened for cyanogenesis and then four (AZ 2327, AZ 2987, AZ 2336 and AZ 3292) were used for further hybridisation and analysis. Additionally, eight sources of subspecies *petrisavii* were screened, including six representing var. *petrisavii* and two of var. *meneghinianum* (Williams et al. 2000). The descriptions of all accessions are presented in Table 1.

Initial screening was carried out on potted plants in an insect-proof glasshouse at Palmerston North. Selected plants (see Results) were crossed by hand using the method of Williams (1954). Mature seeds were harvested 3–6 weeks after pollination. Families of progeny seedlings were grown from scarified seed in plastic trays of standard potting mix and tested for cyanogenesis and shikimate dehydrogenase alleles. Where necessary, selected seedlings were transplanted into individual pots for subsequent crossing and the production of further generations.

Cyanogenesis testing was done using a scaled-down version of the picrate paper method (Corkill 1940). One young leaflet and 20 µl of toluene were placed in the bottom of a 1.5-ml microfuge tube with a small square of filter paper, soaked in picric acid solution, fitted below the lid. Tubes were incubated at 37°C overnight and then the filter paper was observed for the change of colour from yellow to red. Where appropriate, plants testing negative were further analysed for the presence of linamarin/lotaustralin by the addition of 10 µl of 0.5% linamarase and for the presence of linamarase by the addition of 20 µl of 0.5% linamarin. The latter test was also conducted by replacing the linamarin with a leaflet from a plant with phenotype *Acli* (linamarin positive, linamarase negative).

Allozymes of SDH were separated using non-denaturing, discontinuous, polyacrylamide gel-electrophoresis (gels 170-mm wide times 160-mm×1-mm thick). The running gels (6% acrylamide), stacking gels (3.4% acrylamide) and the tank buffer were according to Sambrook et al. (1989). Electrophoresis was conducted at 4°C for 1 h at 50 mA followed by 5 h at 20 W constant power. Staining was according to Tanksley and Rick (1980). Gels were dried overnight and used as records for interpretation of data.

Results

Screening for acyanogenic phenotypes

Results of screening 47 plants of subspecies *petrisavii* and 81 plants of subspecies *nigrescens* for cyanogenesis are given in Table 1. Only one plant (subspecies *nigrescens* AZ 3292-1) was found to be acyanogenic. On analysis it proved to be Ac li in phenotype. Assuming the two locus-two allele model from white clover (Corkill 1942) possible genotypes were *Ac*, *Ac*; *li*, *li* or *Ac*, *ac*; *li*, *li*. This plant and its progeny were analysed to test: (1) whether the two-locus model was appropriate to describe the inheritance of cyanogenesis in *T. nigrescens*, and (2) whether there was evidence of linkage between *Li* and *Sdh*.

Because this was the first observation of a recessive allele in this species, a further 52 plants from the population AZ 3292 were screened for cyanogenesis. One other plant showed an acyanogenic (Ac li) phenotype, indicating the presence of homozygous recessive *li*. To detect heterozygotes in AZ 3292, five plants were crossed with a double-recessive tester (*ac*, *ac*; *li*, *li*) derived from AZ

Table 1 Origins of the accessions of the *T. nigrescens* subspecies used and the results of cyanogenesis testing

Accession	Origin	Number of plants Ac Li	Number of plants Ac li
	Subspecies *nigrescens*		
AZ 751	Inst. Bot., Coimbra Univ., Portugal	7	0
AZ 2225	Mig Gen Veg, Uni Venanzo, Terni, Italy	4	0
AZ 2327	Inst Gen Pl Br, Prague, Czech Republic	2	0
AZ 2336	Research Inst. for Fodder Plants Ltd, Troubsko, Czech Republic	4	0
AZ 2923	Uni Bot Have, Copenhagen, Denmark	4	0
AZ 2987	Caldas da Rainha, Estremadura, Portugal	10	0
AZ 3040	SARDI, Adelaide, Australia (ex Kea Is, Greece, SA8428 ex CPI 116453)	4	0
AZ 3041	SARDI, Adelaide, Australia (ex Kea Is, Greece, SA8429 ex CPI 116453)	3	0
AZ 3094	SNES, Serv Bot Dept Agric, Rouen, France	2	0
AZ 3095	SNES, Serv Bot, La Miniere, Versailles, France	4	0
AZ 3096	Increase of AZ 751	1	0
AZ 3281	Alges Estremadura, Estac Agron Nac, Oeiras, Portugal	7	0
AZ 3288	SNES, Serv Bot Dept Agric, Rouen, France	4	0
AZ 3289	Inst Sup Ag, Lisbon, Portugal	4	0
AZ 3290	Increase of AZ 2336	6	0
AZ 3291	Dry meadow, Benavente, Ribatejo, Portugal	8	0
AZ 3292	Wasteground, Perugia, Italy	3	1
AZ 3295	Prof K.Hammer, Gatersleben, Germany (ex Portugal)	4	0
	Subspecies *petrisavii* var. *petrisavii*		
AZ 125	CSIRO, Canberra, Australia (CPI 21878 ex Washington, USA)	3	0
AZ 3092	Increase of AZ 125	6	0
AZ 3093	Increase of AZ 125	4	0
AZ 3257	Dr G. Pederson, USDA-ARS, Mississippi (cv. Segrest)	5	0
AZ 3276	Prof. N.Taylor, Univ Kentucky, USA (S-28-1)	5	0
AZ 3287	Increase of AZ 125	4	0
	Subspecies *petrisavii* var. *meneghinianum*		
AZ 1308	USDA Fed Pl Introd Stn, Chico, California	16	0
AZ 3296	Prof K.Hammer, Gatersleben, Germany (ex Turkey)	4	0

3292-1 (see below). Two of these five plants carried recessive alleles in heterozygous form, one *li* and a second plant *ac*. Thus recessive alleles of both genetic loci were present at easily detectable frequencies in this population.

Characterisation of plants for SDH

Initial screening of *T. nigrescens* for SDH showed that it was polymorphic with at least five alleles, each allele being represented by a band in a different position on the gels. The bands were designated as alleles-A, B, B*,C and D (*Sdh-A, B, B*, C, D*) from anode to cathode. (The *Sdh-B** band was in a similar position to *Sdh-B*, but clearly distinguishable from it).

AZ 3292-1 was homozygous for *Sdh-C*. Other plants screened for SDH included AZ 2327-2, which was heterozygous (*Sdh-B, Sdh-C*), AZ 2987-2 (*Sdh A, Sdh-D*) and AZ 2336-4 (*Sdh-B*, Sdh-B*).

Construction of *Ac, -*; *li li*; *Sdh-C, Sdh-C* and *ac, ac*; *li, li* testers

Construction of a double-recessive tester for the *Li* locus was started by hybridising AZ 3292-1 (*Ac, -*; *li, li*; *Sdh-C, Sdh-C*) with AZ 2987-2 (*Ac, -*; *Li, -*; *Sdh-A, Sdh-D*). Two seedlings from this cross were selected at random and pair-crossed, and the progeny screened. Among five progeny plants which reached flowering, one had the phenotype Ac; li; Sdh-C. Subsequent analysis of this plant showed that its genotype was *Ac, ac*; *li, li*; *Sdh-C, Sdh-C*. This plant was used as the initial tester. The origin of both the *ac* and *li* alleles could be traced to one plant (AZ 3292-1, see below).

Plants of genotype *ac, ac*; *li, li* arose from mating the above tester with plants of genotype *Ac, ac*; *Li, li* derived from crossing AZ 3292-1 with plants of the common type (*Ac, Ac*; *Li, Li*). These were produced in useful numbers from inheritance studies presented here (Tables 2 and 5).

Inheritance of linamarase and SDH in three accessions

Plant AZ 2336-4 (*Ac, -*; *Li, -*; *Sdh-B, Sdh-B**) was crossed with AZ 3292-1 (*Ac ac, li li*; *Sdh-C, Sdh-C*). One progeny plant (phenotype Ac; Li: Sdh-B*, Sdh-C) was crossed with the tester (*Ac, -*; *li, li*; *Sdh-C, Sdh-C*) and the results of screening 73 progeny plants are presented in Table 2. The *Li*:*li* segregation ratio was 38:35 (1:1), while *Ac*:*ac* segregated 49:24 (close to 2:1 but not significantly different from 3:1, χ^2=2.42, 1 *df*, P>0.05), indicating that both parents carried the *ac* allele. There was strong evidence of linkage between *Li* and *Sdh* (χ^2=50.97, *P*<0.01) with a frequency of non-parental combinations of 6/73 (8.2%).

Four plants from the cross AZ 2327-2 (*Ac, -*; *Li, -*; *Sdh*-unknown) ×AZ 3292-1 (*Ac ac, li li*; *Sdh-C, Sdh-C*) were grown. Three proved to be Ac Li; Sdh-B, Sdh-C in phenotype. One of these was crossed with the tester (*Ac ac, li li, Sdh-C, Sdh-C*). Progeny test results are given in Table 3. Results indicated that the derived plant was *Ac, Ac*; *Li, li*; *Sdh-B, Sdh-C*. *Li*/*li* assorted 37:31, i.e. 1:1 as expected (χ^2=0.53 *P*>0.5), and B/C 36:32 (1:1 χ^2=0.24 *P*>0.5). The frequency of non-parental combinations of *Li* and *Sdh* alleles was 3/68 (4.4%).

From the cross AZ 2987-2×AZ 3292-1, two plants were crossed with the tester and the resulting progenies analysed. In the first cross (Table 4), segregations of *Li*:*li* and *Sdh-D*/*Sdh-C* alleles were close to 1:1, as expected. Non-parental combinations of *Li* and *Sdh* alleles occurred with a frequency of 10/75 (13.3%). In the second family (Table 5), segregation of *Ac*/*ac* was 64:17 (not significantly different from 3:1), while *Li*/*li* and *Sdh-D*/*Sdh-C* segregated 1:1. The frequency of non-parental combinations of *Li* and *Sdh* alleles was 18/81 (22.2%). The frequency of non-parental combinations of *Li-Sdh* from these two related families was 28/156 (17.9%). There was no significant heterogeneity between the linkage estimates from the two families (χ^2=1.23, *P*>0.2).

Table 2 Phenotype frequencies resulting from the cross (AZ 2336-4×AZ 3292-1)-1 (*Ac ac, Li li, Sdh-B*, Sdh-C*)×tester (*Ac, ac*; *li, li*; *Sdh-C, Sdh-C*)

Parental Li B*		Recomb. Li C		Recomb. li B*		Parental li C	
Ac	ac	Ac	ac	Ac	ac	Ac	ac
26	10	1	1	1	3	21	10

Table 3 Phenotype frequencies resulting from the cross (AZ 2327-2×AZ 3292-1)-2 (*Ac Ac, Li li, Sdh-B, Sdh-C*)×tester (*Ac, ac*; *li, li*; *Sdh-C, Sdh-C*)

Parental Ac Li B C	Recomb. Ac Li C C	Recomb. Ac li B C	Parental Ac li C C
35	2	1	30

Table 4 Phenotypic frequencies resulting from the cross (AZ 2987-2×AZ 3292-1)-2 (*Ac, -*; *Li, -*; *C, D*)×tester (*Ac, ac*; *li, li*; *C, C*)

Parental Ac Li C D	Recomb. Ac Li C C	Recomb. Ac li C D	Parental Ac li C C
28	6	4	37

Table 5 Phenotypic frequencies resulting from the cross (AZ 2987-2×AZ 3292-1)-4 (*Ac, -*; *Li, -*; *Sdh-C, Sdh-D*)×tester (*Ac, ac*; *li, li*; *Sdh-C, Sdh-C*)

Parental Li D		Recomb. Li C		Recomb. li D		Parental li C	
Ac	ac	Ac	ac	Ac	ac	Ac	ac
25	5	9	1	5	3	25	8

A heterogeneity test of the linkage estimates from the three accessions of diverse origin showed no signficant difference among the estimates that averaged 12.2% from an overall sample of 297 individual plants.

Discussion

Polymorphism for cyanogenesis in *T. nigrescens*

There are no previous reports of polymorphism in this species, it having been described previously as monomorphic for cyanogenesis (Kakes and Hakvoort 1994). An unexpected feature of this study was, therefore, the discovery of recessive alleles at both the *Ac* and *Li* loci in one population and indeed in one plant (AZ 3292-1 *Ac*, *ac*; *li*, *li*) among the first four plants grown from a germplasm accession collected in Italy. Subseqent tests revealed that this population was polymorphic at both loci. *T. nigrescens* therefore shares with *T. repens* the existence of populations with genetic variation at both cyanogenic loci. In *T.repens*, the relative frequencies of dominant and recessive alleles at both loci are associated with the environment (Daday 1965). *T. nigrescens* also occurs over a considerable range of environments and it may be possible in the future to analyse in this species any associations of allele frequencies and environments.

Genetic analysis of *Ac* and *Li*

Both *Ac*/*ac* and *Li*/*li* segregated as expected from independently assorting bi-allelic loci with dominance. The observed inheritance pattern is consistent with the two-locus model adopted for *T. repens* and supports the hypothesis that *T. nigrescens* and white clover have a common ancestry.

Linkage of the *Li* and *Sdh* loci

Linkage of *Li* and *Sdh* was observed in all four families analysed, representing three accessions of diverse origins (obtained independently from Portugal and the Czech Republic). This result clearly demonstrated that the linkage was not restricted to one population but is apparently a characteristic of the subspecies as a whole. The families analysed were relatively small and, while the recombination frequencies did not show significant heterogeneity, only further analysis of larger families will determine whether the linkage distance is the same in populations from widely separated regions.

Comparison with white clover

Williams et al. (1998) showed that *Li* and *Sdh* were linked in one of the two genomes of white clover while the other genome lacked *Li*. It is likely that the ancestral species that donated the *Li*-carrying genome was a cyanogenic species and that *T. nigrescens* is a candidate. The synteny between *T. repens* and *T. nigrescens* tends to support this hypothesis. The mean *Li-Sdh* recombination frequency for *T. nigrescens* (12.2%) compares with the white clover distance of 6.0±2.0 cM (Williams et al. 1998). To-date, the evidence of synteny between these two *Trifolium* species is limited to this study, which gives no indication of the comparative directionality of the linkages. Synteny over short chromosome regions even of relatively distantly related taxa is widely documented and occurs, for example, in grass genera that have been separated by over 60 million years of independent descent (Doebley et al. 1990).

The question of whether *T. nigrescens* subsp. *nigrescens* is a direct ancestor of, and has donated its genome to, *T. repens* is not answered by this work. However, other research based on comparative physical mapping of rDNA (Ansari et al. 1999; Williams et al. 2001) and analysis of centromeric DNA (Ansari et al. submitted) has indicated that subsp. *nigrescens* is unlikely to be a direct ancestor of *T. repens.* Subspecies *petrisavii*, on the other hand, is a more likely candidate. Lack of polymorphism in the SDH of subspecies *petrisavii* currently prevents closer analysis along these lines. It is concluded that *T. nigrescens* ssp. *nigrescens* and *T. repens* show synteny in the *Li-Sdh* chromosome region and share a common ancestral lineage, but this does not necessarily indicate that *T. nigrescens* ssp. *nigrescens* has donated a genome to white clover.

Acknowledgements The authors thank John Caradus and Derek Woodfield for helpful comments on the manuscript. This research complies with the current laws of New Zealand.

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