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A molecular marker linkage map of tetraploid alfalfa (*Medicago sativa* L.)

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Abstract A genetic linkage map was constructed for an F_1 genotype of auto-tetraploid alfalfa (*Medicago sativa* L.) using two backcross populations of 101 individuals each and 82 single-dose restriction fragments segregating in each population. The percentages of marker loci deviating from Mendelian ratios were considerably less than reported for inbred diploid mapping populations (4–9% compared to 18–54%), probably due to the greater buffering capacity of autotetraploids against the effects of deleterious recessive alleles. Four homologous coupling-phase cosegregation groups were detected for seven of the eight linkage groups of diploid alfalfa and aligned using probes in common. No cosegregation groups were found for linkage group 7 due to the lack of polymorphisms in this cross. A composite map was generated by integrating the four homologous cosegregation groups and consisted of 88 loci on seven linkage groups covering 443 cM. The locus map-orders and distances were in general agreement with those found in diploid alfalfa. The mapping population segregates for winterhardiness, fall dormancy, and freezing tolerance; and the map will be used to locate genomic regions affecting these traits.

Key words *Medicago sativa* · Tetraploid · Linkage mapping · RFLPs · SDRFs

Introduction

Alfalfa, the most important perennial forage crop in the world, is an outcrossing autotetraploid (2n=4*x*=32) and is included in the *Medicago sativa* complex along with diploid and tetraploid relatives. Several molecular genetic linkage maps of alfalfa have been created using diploid populations in order to avoid the complicated tetrasomic inheritance and linkage relationships of tetraploids (Brummer et al. 1993; Kiss et al. 1993; Echt et al. 1994; Tavoletti et al. 1996). Yu and Pauls (1993b) detected nine linkages between RAPD markers segregating in tetraploid alfalfa; however, a detailed linkage map of tetraploid alfalfa has not been reported.

Since alfalfa chromosomes predominately pair as bivalents (Bingham and McCoy 1988), random chromosome segregation with no double reduction can be assumed. Wu et al. (1992) proposed the use of single-dose restriction fragments (SDRFs) to greatly simplify linkage mapping in polyploid species whose chromosomes pair as bivalents. SDRFs are restriction fragments that are assumed to be present in a single dose and segregate 1:1 (present/absent) in gametes of heterozygous plants. The segregation ratios and linkage equations for SDRFs are equivalent to diploids for the detection of linkages in coupling. Since the power to detect repulsion-phase linkages between SDRFs in tetraploids is much lower than for diploids, a coupling-phase linkage group (cosegregation group) is generated for each homologous chromosome (Grivet et al. 1996). Homologous cosegregation groups can be identified using multiple SDRFs generated by highly polymorphic DNA clones that detect single loci (Liu et al. 1998). Although this method only uses data from simplex loci, duplex loci can be incorporated into the final map by using the equations of Yu and Pauls (1993b).

Four genetic linkage maps have been constructed in diploid alfalfa, which utilized RFLP, RAPD, isozyme, seed protein and morphological marker loci (Brummer et al. 1993; Kiss et al. 1993; Echt et al. 1994; Tavoletti et al. 1996). Three of the four maps identify eight linkage groups corresponding to the basic chromosome number of *x*=8, and the fourth detects ten groups that should coalesce to eight with more markers. The maps of Echt et al. (1994) and Tavoletti et al. (1996) share 35 RFLP clones and have highly conserved marker orders. Probes

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are not shared between the other maps. The maps have been used to locate genes controlling flower color, dwarfness, sticky leaves (Kiss et al. 1993), seed proteins, nodulation factors (Kiss et al. 1997), and a unifoliate leaf, cauliflower head mutation (Brouwer and Osborn 1997a).

One of the most powerful uses of molecular marker linkage maps in crop plants is the dissection of quantitative traits into discrete genetic loci. Important traits at the tetraploid level could be studied in diploids if genetic variation for the trait can be identified at the diploid level. Alternatively, tetraploid germ plasm can be reduced to the diploid level for evaluation and then useful genes transferred to the tetraploid level for incorporation into breeding programs. However, general combining abilities for forage yield and fertility are not correlated when studied in isogenic diploid and tetraploid alfalfa, suggesting that genes affecting quantitative traits may have different affects at the two ploidy levels (Groose et al. 1988). Thus, mapping at the tetraploid level may be more informative for some quantitative traits, and would eliminate the need for ploidy level manipulations.

This paper reports the first detailed linkage map of tetraploid alfalfa. We utilized RFLP probes from diploid maps allowing us to compare diploid and tetraploid maps. The tetraploid mapping population segregates for traits associated with winterhardiness, and the maps will be used to locate QTLs affecting these traits.

Materials and methods

Plant materials

Two tetraploid plants, Blazer XL 17 (B17) and Peruvian 13 (P13), were used to develop two backcross populations. B17 was a very fall-dormant, cold-tolerant, and winter hardy genotype derived from the cultivar Blazer XL. P13 was a non-fall-dormant, cold-sensitive, and winter-sensitive genotype from PI 536535 which represents the original Peruvian germ plasm source of North American cultivated alfalfa and contains unique RFLP diversity that separates it from the main germ-plasm sources of cultivated alfalfa (Kidwell et al. 1994a). A single F_1 hybrid of the cross B17×P13, confirmed by inherited RFLP alleles, was backcrossed as a female to each parent using vacuum emasculation to prevent self-pollination. One hundred and one progeny for each population were confirmed to be backcross progeny based on RFLP genotypes and were used for mapping.

Detection and analysis of segregating restriction fragments

The procedures used for DNA extraction, Southern blotting, isolation and labeling of probe inserts, and Southern hybridization were described in Kidwell et al. (1994b) using the modifications described in Brouwer and Osborn (1997b). DNAs were digested with *Eco*RI, *Eco*RV, *Hin*dIII, and *Xba*I. The RFLP probes originated from several sources. The 20 UWG, seven HG, and 41 VG clones developed at the University of Wisconsin and the 15 MTSC clones obtained from Dr. Tom McCoy (Montana State University) were used previously to map RFLP loci in a diploid backcross population from a cross of two Cultivated Alfalfa at the Diploid Level (CADL) plants (Echt et al. 1994) and a diploid F_1 population from a cross of a CADL plant and a *M. sativa* spp. *falcata* diploid (Tavoletti et al. 1996). The 38 UGAC clones were used previously to map RFLP loci in an F_2 population from a cross of a CADL plant and a *M. sativa* spp. *falcata* plant (Brummer et al. 1993) and were obtained from Dr. Joe Bouton (University of Georgia).

For each clone, unique fragments that were present in one parent, absent in the other parent, and segregating in the backcross progeny were scored independently as dominant markers. Fragments were labeled with the clone name and a suffix of p(x) for fragments originating from P13 and mapped in the backcross to B17 and b(x) for fragments originating from B17 and mapped in the backcross to P13, where x=1, 2, and 3 designated different fragments from a parent detected by that clone.

Only fragments segregating as SDRFs were mapped in each backcross. The expected segregation ratio for an SDRF in a backcross population is 1:1 compared to 5:1 for a duplex locus (Wu et al. 1992). Fragments segregating at a presence to absence ratio of less than 2.24:1 were considered SDRFs. This ratio gives equal χ^2 values for both 1:1 and 5:1 hypotheses (Mather 1951). Each SDRF locus was also tested by χ^2 analysis to identify those loci showing significant (P<0.05) deviation toward absence of the fragment.

Two other segregation analyses were performed when data were available. A ratio of 3 present:1 absent for fragments in the backcross to the parent contributing the allele was tested by χ^2 analysis for clones evaluated in both backcross populations. Clones that detected two unique SDRFs donated by one parent to the F_1 could also be tested by χ^2 analysis for fit to a 1:4:1 ratio of 0, 1, or 2 alleles from that parent in the backcross to the other parent.

Linkage analysis

Linkage maps were generated separately for each backcross population. The SDRFs in each backcross were analyzed as an "F_2 backcross" using MAPMAKER v2.0 (Lander et al. 1987) for MacIntosh. The SDRFs, scored as dominant loci, were entered with A for the absence of a fragment and H for the presence of a fragment. Loci were grouped by decreasing the LOD score from 9 to 3 in 2-unit increments. Orders within each group were determined by multipoint analysis using standard MAPMAKER methods, and the most-likely order selected. Loci were sorted according to this order, and double-crossover events were rechecked for scoring errors on the original autoradiographs. All maps were generated using the Kosambi map function. Since the SDRF method only detects coupling-phase linkages (Grivet et al. 1996), up to four homologous cosegregational groups can be detected for each linkage group. The cosegregational groups were aligned using SDRFs detected by the same probe and information from the diploid alfalfa maps. Finally, loci with duplex ratios were tested for linkage to each cosegregation group using the formulas described in Yu and Pauls (1993b).

A composite map for each linkage group was constructed to integrate map information from the homologous cosegregation groups using Map+ (Collins et al. 1996), a free software package developed to build consensus genetic maps based on multiple pairwise information. Recombination distances and LOD scores for all pairwise combinations of SDRFs on each cosegregation group were generated using the LODS command of MAPMAKER. For each linkage group, cosegregation groups with the most paired SDRFs were joined first. Diploid RFLP maps were used for reference when only one shared pair of SDRFs joined the cosegregation groups.

Results

Segregation analysis

A total of 122 probes was surveyed in the two parents, the F_1, and a subset of backcross genotypes using four enzymes: *Eco*RI, *Eco*RV, *Hin*dIII, and *Xba*I. Nine probes did not detect polymorphism, and six probes detected polymorphic fragments between the parents that were fixed in the backcross progeny. Eleven probes detected polymorphic fragments between the parents that were not inherited by the F_1 plant but segregated in one of the

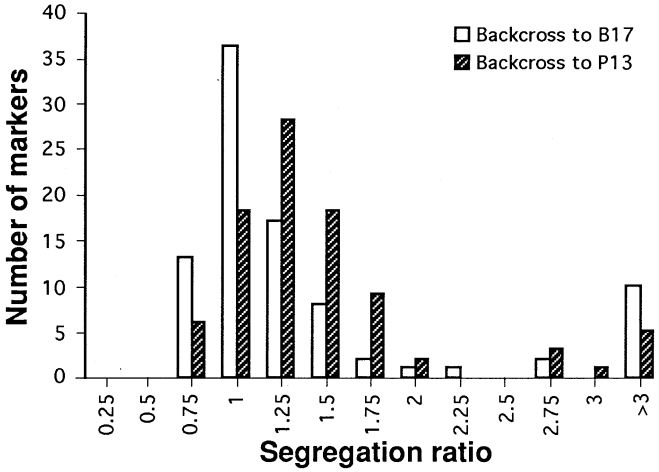

Fig. 1 Distribution of segregation ratios (presence:absence) of restriction fragments from the non-recurrent parent segregating in two backcross populations. Fragments with a ratio greater than 2.24:1 were considered to represent duplex loci and were not included in SDRF mapping

backcross progeny because the fragment was heterozygous in the recurrent parent. The remaining 96 probes detected at least one fragment in the F_1 that segregated in one of the backcross populations and these were evaluated in the full-backcross populations using all enzymes that detected unique segregating fragments.

Ninety one segregating fragments detected with 68 probes, and 89 segregating fragments detected with 65 probes, were scored in the backcrosses to B17 and P13, respectively. Using Mather's criterion to distinguish 1:1 vs 5:1 segregation ratios, 81 fragments from B17 and 80 fragments from P13 fit the 1:1 presence vs absence ratio expected for simplex alleles, while ten fragments from B17 and nine fragments from P13 fit the 5:1 presence vs absence ratio expected for duplex allele ratios (Fig. 1). However, the presence of four unique SDRFs in some backcross individuals indicated that allele *vg1b10p1* in the backcross to B17, and alleles *vg1b10b1* and *vg1h10b1* in the backcross to P13, were simplex alleles with segregation distortion despite ratios that supported duplex inheritance. Thus, 82 SDRFs were retained for mapping in each backcross and were derived from 62 probes in B17 and from 63 probes in P13.

Only 3 of 82 SDRFs (4%) in each population were skewed toward values lower than 1 present:1 absent, and, as mentioned previously, one SDRF in B17 and two SDRFs in P13 were significantly skewed toward higher duplex type ratios (Table 1). The average ratio of presence:absence for SDRFs was 0.99:1 in the B17 population and 1.15:1 in the P13 population, the latter of which was significantly (P<0.01) different from 1:1. Three of forty three B17 alleles (8%) and 5 of 48 P13 alleles (10%) that were scored in the backcross to the parent contributing the allele deviated significantly from the expected 3:1 ratio (P<0.05; Table 1). Four and five of 16 loci in the B17 and P13 backcross populations, respectively, that were scored for two non-recurrent parent alleles deviated significantly (P<0.05) from the 1:4:1 ratio

Table 1 Single-dose restriction-fragment alleles that deviated significantly (P<0.05) from expected segregation ratios for presence (**P**) and absence (**A**) in one or both backcross populations

Allele	Linkage group	Recurrent parent	No. individ.		Expected ratio (P:A)	χ^2
			P	A		
Backcross to parent not contributing the allele						
uwg284p1	1A	B17	38	62	1:1	5.76*
vg1f12p1	2B	B17	36	57	1:1	4.74*
vg1b10p1	8B	B17	87	12	1:1	56.82***
vg1b10p2	8A	B17	35	64	1:1	8.49**
ugac393b1	5D	P13	40	60	1:1	4.00*
vg1h10b1	5D	P13	76	25	1:1	25.80***
vg1b10b1	8D	P13	72	25	1:1	22.80***
vg1b10b2	UL	P13	37	56	1:1	3.88*
vg2c2b1	UL	P13	38	63	1:1	6.19*
Backcross to parent contributing the allele						
vg1h6b2	4D	B17	99	2	3:1	28.50***
vg1h10b1	5D	B17	81	15	3:1	4.50*
vg2c1b2	UL	B17	87	14	3:1	6.68**
mtsc9p1	1A	P13	64	37	3:1	7.29**
vg2b9p1	1B	P13	90	14	3:1	7.38**
mtsc14p1	3B	P13	86	15	3:1	5.55*
ugac671p1	4A	P13	86	15	3:1	5.55*
vg2c2p1	6A	P13	88	13	3:1	7.92**

Table 2 Loci having two non-recurrent parental alleles that deviated significantly (P<0.05) from a 1:4:1 ratio in one or both backcross populations

Locus	Linkage group	Allele dose[a]			χ^2
		0	1	2	
Backcross to B17					
ugac671	4	19	54	26	8.03*
ugac191	8	30	44	16	19.33***
ugac540	8	17	30	20	14.85***
vg1b10	8	4	68	27	16.21***
Backcross to P13					
mtsc9	1	9	64	28	11.22**
ugac85	3	19	50	27	11.19**
vg1g9	5	8	67	25	8.68*
mtsc6	8	16	59	26	6.06*
ugac540	8	12	24	13	6.96*

[a] Doses of the non-recurrent parent alleles in the backcross to the parent not contributing the SDRFs: 0 doses, neither SDRF allele is present; 1 dose, one of the two SDRF alleles is present; and 2 doses, both of the SDRF alleles are present

(Table 2). Loci exhibiting segregation distortion were located on linkage groups 1 and 5 in the backcross to P13 and on linkage group 8 in both backcrosses (Fig. 2).

Linkage analysis

Grouping of the 82 SDRFs from each population into coupling-phase linkage groups corresponding to the eight sets of four homologous chromosomes was investigated by dropping the LOD score from 9 to 3 in 2-unit increments. Decreasing the LOD score from 5 to 3 erroneously joined two homologous linkage groups containing four

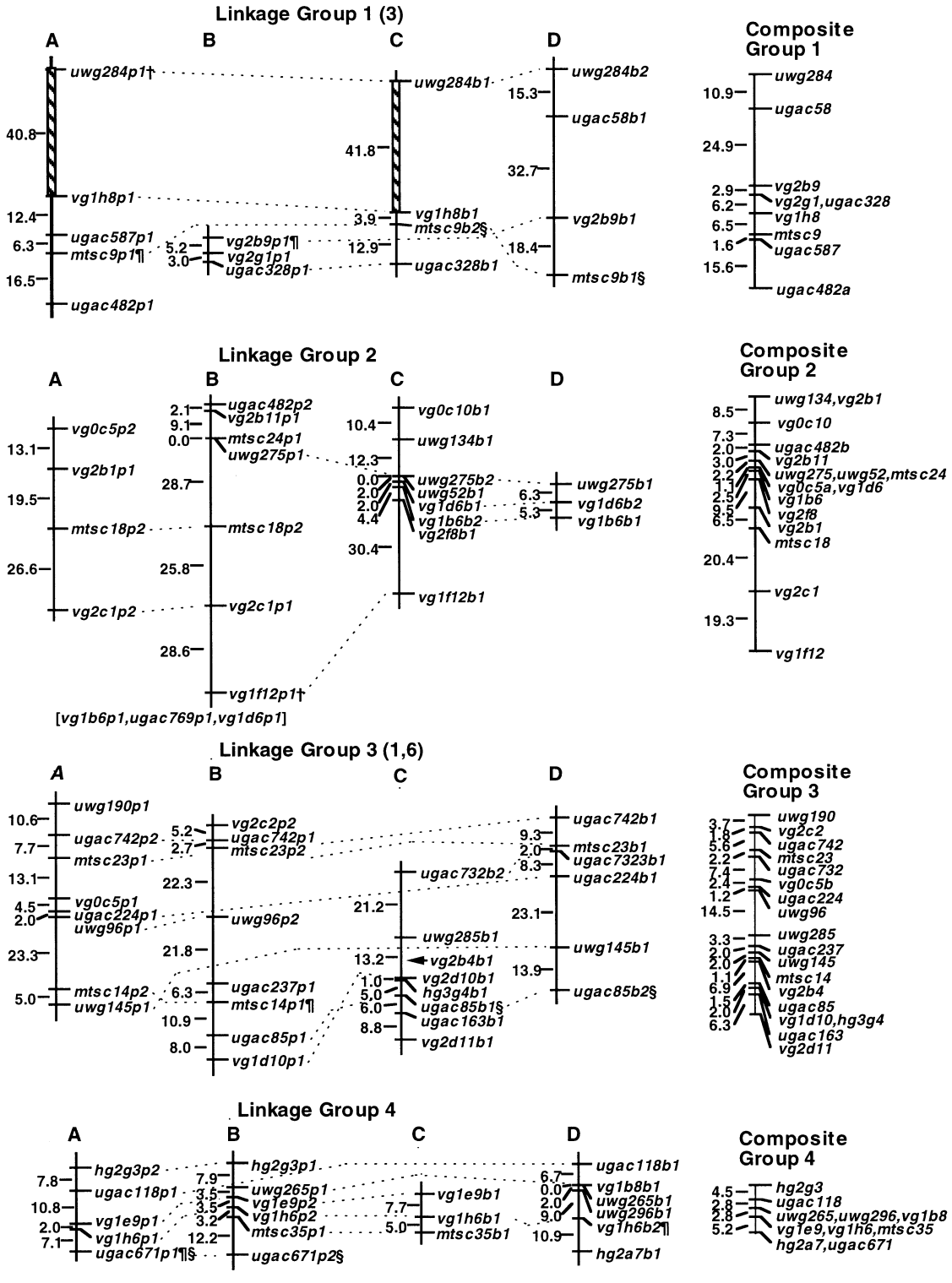

Fig. 2 Molecular marker linkage map of tetraploid alfalfa (*M. sativa* L.) The four homologous cosegregation groups for each linkage group in the F_1 were mapped in the backcross to Blazer XL 17 (*A and B*) and the backcross to Peruvian 13 (*C and D*). Map distances are shown in centiMorgans using the Kosambi mapping function, and linkage groups are numbered according to Tavoletti et al. (1996) and Echt et al. (1994) with corresponding groups from Brummer et al. (1993) shown in *parenthesis*. The SDRFs are *labeled* with the RFLP probe as a prefix and a suffix designating that the SDRF originated from the Blazer XL 17 parent (*b1* or *b2* or *b3*) or the Peruvian 13 parent (*p1* or *p2*). *Dashed lines* connect SDRFs detected by the same probe and mapped to the same linkage group. *Hatched segments* indicate linkages with LOD scores <3.00 and Theta >0.40, but were presumed to be linked because of linkages identified for other alleles detected with the same probe in homologous cosegregational groups. † indicates SDRF loci that deviated significantly from a 1:1 ratio (P<0.05) with a deficiency in genotypes having the SDRF; ‡ indicates SDRFs that are simplex based on parental genotypes but fit the duplex criteria; ¶ indicates loci that deviated significantly (P<0.05) from a 3 present:1

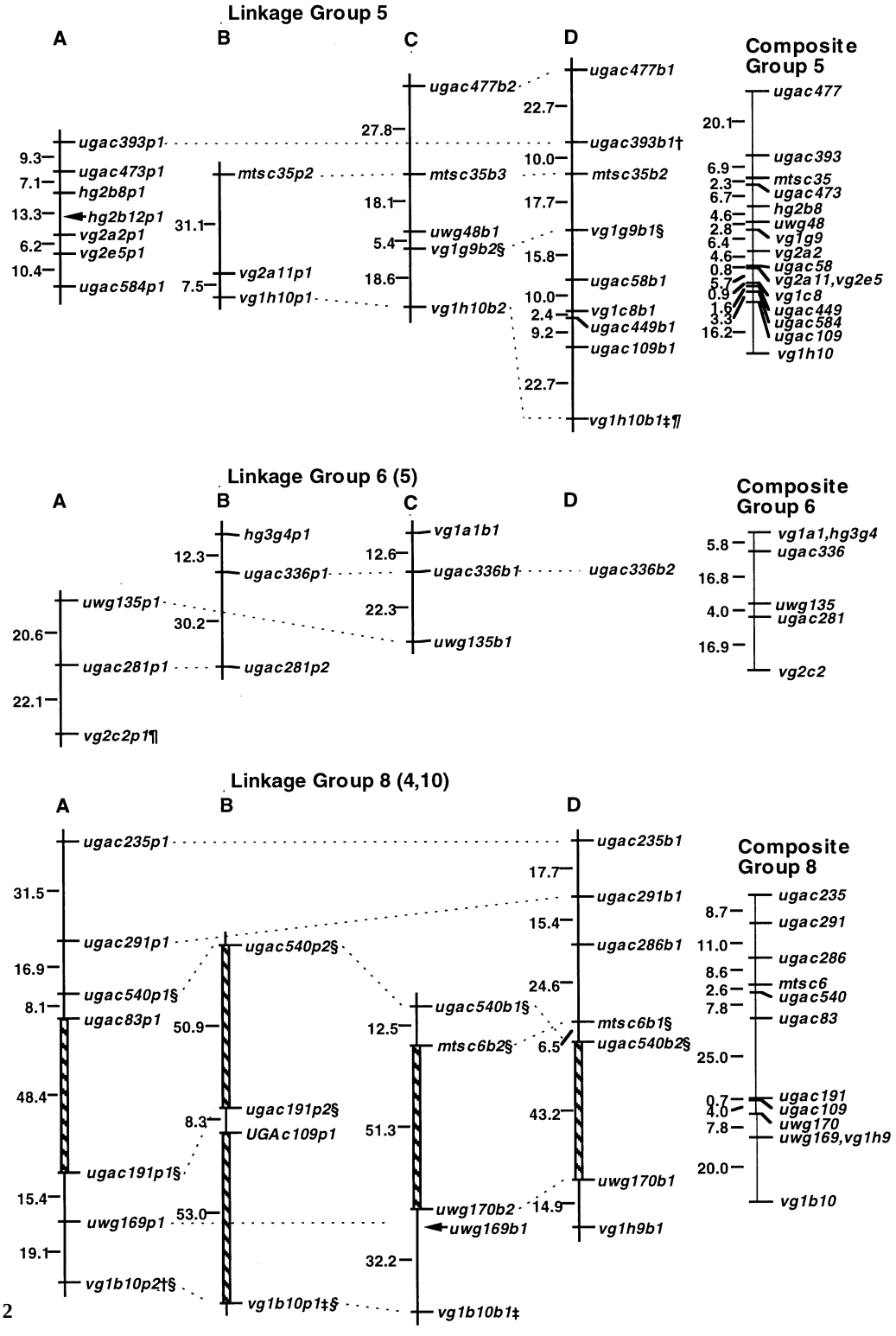

Fig. 2

absent ratio in the backcross to the parent contributing the allele, and § indicates loci that deviated significantly ($P<0.05$) from a 1:4:1 ratio of 0, 1, or 2 alleles from the non-recurrent parent in the backcross to the parent not contributing the allele. Three diallele duplex loci that show linkage to both groups 2A and 2B are listed in *parenthesis* below the groups. *Arrows* indicate the approximate positions of two SDRFs that were not included in map-distance estimates because they caused severe map inflation. The composite map, based on integrating the homologous cosegregation groups of SDRFs from two backcross populations, is shown to the right of the set of four homologous cosegregation groups. Multiple marker loci detected by the same probe were labeled with a *letter suffix* in the composite map

SDRFs detected by two probes with simple banding patterns (cosegregation groups 3 C and 3D in Fig. 2), and also introduced several linkages between SDRFs detected by probes which detected unlinked loci in previous maps (Brummer et al. 1993; Echt et al. 1994; Tavoletti et al. 1996). Using a LOD of 5 produced 15 cosegregation groups from B17 and 16 cosegregation groups from P13. Cosegregation groups were further organized based on SDRFs detected by the same probe and SDRFs detected by probes linked in diploid maps. This process resulted in seven basic sets corresponding to seven of the eight linkage groups of Tavoletti et al. (1996) (Fig. 2). In each backcross, three SDRFs that were linked to existing cosegregation groups with a LOD of 3 were added to the map since the probes used also detected SDRFs on other homologous cosegregation groups or were mapped to the same linkage group in diploid alfalfa (Brummer et al. 1993; Echt et al. 1994; Tavoletti et al. 1996). These criteria were also used to accept linkages below LOD 3 for SDRFs on linkage groups 1 and 8 (Fig. 2). This resulted in the consolidation of seven cosegregation groups into four homologues for linkage group 8. Three of the loci with duplex segregation ratios showed linkage to both groups 2 A and 2B (Fig. 2). Insertion of two SDRFs caused large map-distance inflation and these were not included in the map; however, their approximate positions are indicated by arrows in Fig. 2. Loci detected by probes from linkage group 7 of Tavoletti et al. (1996) and linkage group 8 of Brummer et al. (1993) were either fixed in the backcross populations or detected duplicate loci segregating on other linkage groups and, thus, this linkage group was not identified.

All homologues in linkage groups 1, 2, 3, 4, and 8 were aligned using two or more pairs of SDRFs detected by the same probe (Fig. 2). Cosegregation-group 5 A of linkage group 5 was aligned to the other homologues so that *vg1g9* and *vg2a2* were close together since they mapped only 7.5 cM apart in diploid alfalfa (Tavoletti et al. 1996). Cosegregation-groups for linkage group 6 were aligned to minimize composite map distances.

A consistent order of loci was observed among homologues of each linkage group. The only inverted locus order occurred for markers UGAC85 and VG1D10 between groups 3B and 3C (Fig. 2). Map distances between loci detected on more than one homologue were also relatively consistent. Exceptions were the interval between *mtsc9* and *vg1h8* which was 18.7 cM on 1 A and 3.9 cM on 1 C, the interval between *uwg275* and *vg1f12* which was 83 cM on 2B and 38.8 cM on 2 C, and the interval between *mtsc35* and *vg1h10* which was 18.6 cM on 5 C and 60.1 on 5D (Fig. 2).

A composite map was generated for each linkage group by integrating information from the four homologous cosegregation groups. This was justified because SDRF mapping in both backcrosses was based on recombination events from the same F_1 plant and marker order was consistent among homologous cosegregation groups. The composite map consisted of 88 loci on seven linkage groups and covered 443 cM (Fig. 2).

Comparison to diploid alfalfa maps

The positions of marker loci in this tetraploid map were highly congruous with those that could be compared in the diploid maps of Echt et al. (1994) and Tavoletti et al. (1996). The exceptions were probe VG2B1, which detected a locus in a different position on linkage group 2 than was reported by Tavoletti et al. (1996), and probe UWG265, which mapped to linkage group 1 in Echt et al. (1994) and Tavoletti et al. (1996) but mapped to linkage group 4 in our tetraploid map. Several differences in map position were observed for probes which detected complex banding patterns and which may correspond to two or more loci (VG1H8 on linkage group 1; MTSC35, VG0C5, and VG1F12 on linkage group 2; HG3G4, VG0C5, and VG2C2 on linkage group 3; HG2G3 on linkage group 4; and HG2B12 on linkage group 5).

By including probes mapped by Brummer et al. (1993), correspondence between some of the linkage groups could be established. Linkage groups 1 and 6 corresponded to linkage groups 3 and 5 of the Brummer et al. (1993) map, respectively. Group 4 included markers from linkage groups 1 and 6 on the Brummer et al. (1993) map, and our group 8 included markers from linkage group 4 along with one of the two markers on group 10. Linkage groups 2, 4, and 5 on our map included markers that map to linkage groups 2, 7 and 9 on the Brummer et al. (1993) map, but no correspondence between particular groups was identified. Probes detecting loci on linkage group 8 of the Brummer et al. (1993) map showed no polymorphism in our populations, suggesting that this group corresponded to linkage group 7 on the Echt et al. (1994) and Tavoletti et al. (1996) maps.

Discussion

Alfalfa carries a high level of deleterious recessive alleles due to its outcrossing nature and the buffering capacity of polyploidy. In diploid maps developed in inbred populations (backcross or F_2), 18 to 54% of the loci had significantly ($P<0.05$) distorted segregation ratios (Brummer et al. 1993; Kiss et al. 1993; and Echt et al. 1994). The loci with distorted segregation ratios tended to cluster together and were skewed toward heterozygotes. This was explained by the exposure of deleterious recessive alleles during inbreeding which caused gametic and/or zygotic selection leading to the skewed ratios of linked marker loci. In contrast, only 9% of the marker loci mapped in a non-inbred alfalfa population showed significant segregation distortion (Tavoletti et al. 1996). The level of inbreeding in tetraploid alfalfa is much lower than in diploid alfalfa at equivalent generations. The average inbreeding coefficient of backcross progeny derived from quadruplex tetraploid parents is 0.08, compared to 0.25 for diploid backcross progeny derived from heterozygous parents and 0.50 for diploid F_2 progeny. In our backcross populations, only 6 of 162 (4%) chi-square tests for segregating SDRF loci and 8 of 91 (9%) chi-square tests for simplex loci segregating in

the backcross to the parent donating the fragment showed significant ($P<0.05$) deviation . Thus, the buffering capacity of autotetraploid segregation may reduce the selection against deleterious recessive alleles that cause distorted segregation ratios in inbred diploid mapping populations.

Wild diploid relatives and/or diploid derivatives of alfalfa, potato (Bonbierbale et al. 1984) and strawberry (Davis and Yu 1997) have been used to construct linkage maps that are believed to represent genomes of cultivated polyploids. We could make direct comparisons between diploid and tetraploid alfalfa maps by using information from probes tested at both ploidy levels. In our map, 52% of the loci have complex banding patterns (more than four fragments) which may represent more than one locus, similar to the 59% found by Brummer et al. (1993) in diploid alfalfa. The order of probes detecting simple patterns (four or fewer fragements) is highly conserved between our map and the maps of Echt et al. (1994) and Tavoletti et al. (1996). The tetraploid map covered 443 cM on seven linkage groups. The diploid maps that utilized some of the same probes covered 467 cM (Brummer et al., 1993), 553 cM and 602 cM (Echt et al. 1994), and 234 cM and 261 cM (Tavoletti et al. 1996). Distances between individual marker loci are also similar in the diploid and tetraploid linkage maps. Thus, locus orders and linkage distances are concordant across the two ploidy levels.

Molecular markers have been used in diploid alfalfa to map genes controlling qualitative traits (Brouwer and Osborn 1997a; Kiss et al. 1997) and a QTL controlling variation in aluminum tolerance (Sledge et al. 1996). These and other associations discovered at the diploid level could be exploited in cultivated alfalfa by using ploidy level manipulations. However, the effects of some alleles, linkage blocks, or epistatic interactions may be different at the two ploidy levels (Groose et al. 1988). Thus, mapping at the tetraploid level may provide more valuable information, especially for breeding applications of QTL information. Yu and Pauls (1993a) reported linkage between a RAPD marker and a gene controlling somatic embryogenesis in tetraploid alfalfa, and QTL mapping in polyploid sugarcane has been demonstrated by association between two SDRFs and brix value (Liu et al. 1998). The B17 and P13 backcross populations used in our study segregate for winterhardiness, fall dormancy, and freezing tolerance. The linkage maps described here will be used to map QTLs controlling these traits in tetraploid alfalfa.

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