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Linkage relationships among molecular markers and storage root traits of carrot (*Daucus carota* L. ssp. *sativus*)

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Abstract A 109-point linkage map consisting of three phenotypic loci (P_1, Y_2, and *Rs*), six restriction fragment length polymorphisms (RFLPs), two random amplified polymorphic DNAs (RAPDs), 96 amplified fragment length polymorphisms (AFLPs), and two selective amplification of microsatellite polymorphic loci (SAMPL) was constructed for carrot (*Daucus carota* L. ssp. *sativus*; $2n = 2x = 18$). The incidence of polymorphism was 36% for RFLP probes, 20% for RAPD primers, and 42% for AFLP primers. The overall incidence of disturbed segregation was 18%. Linkage relationships at a LOD score of 4.0 and $\theta = 0.25$ indicated 11 linkage groups. The total map length was 534.4 cM and the map was clearly unsaturated with markers spaced at 4.9 cM. AFLP P6B15 was 1.7 cM from P_1, AFLP P1B34 was 2.2 cM from Y_2, and AFLP P3B30XA was 8.1 cM from *Rs*.

Key words Genetic map · RFLP · AFLP · RAPD · SAMPL · *Daucus carota* L. ssp. *sativus*

Introduction

Cultivated carrot (*Daucus carota* L. ssp. *sativus*; $2n = 2x = 18$) is grown worldwide, ranks among the top ten economically important vegetables in the United States, and is consumed either fresh or processed. Its genome size [473 Mbp, 0.98 pg per haploid genome (Arumuganathan and Earle 1991)] is four times that of *Arabidopsis*, equal to that of rice, half that of tomato, and one-fifth that of corn. About 75 phenotypic loci have been reported (Simon 1984, 1996) including P_1, Y_2 and *Rs*. P_1 and Y_2 condition purple and yellow root pigmentation, respectively, in contrast to their non-purple p_1/p_1 and orange y_2/y_2 counterparts (Simon 1996). *Rs* conditions the accumulation of glucose and fructose in the storage root, in contrast to *rs*/*rs* carrots which store sucrose (Freeman and Simon 1983).

Molecular maps have been used for genome comparisons (e.g., Bonierbale et al. 1988), cloning (e.g., Martin et al. 1993) and marker-assisted selection (MAS) (e.g., Edwards 1992; Stuber 1995) for many plants, but carrot (Schulz et al. 1994) and celery (*Apium*) (Huestis et al. 1993) are the only two Apiaceae (Umbelliferae) with genetic maps. Molecular maps of progeny from each of four selfed carrot plants (Schulz et al. 1994) had 5–8 linkage groups with 19–26 markers (phenotypic loci, isozymes, RFLPs, RAPDs) in each map. Amplified fragment length polymorphisms (AFLPs), simple sequence repeats (SSRs), and the selective amplification of microsatellite polymorphic loci (SAMPL) are the other useful molecular markers but they have not been applied to carrot. AFLP markers have been mapped in several crops [e.g., barley (Becker et al. 1995), tomato (Thomas et al. 1995)]. SAMPL combines the advantageous features rendered by SSRs with the procedural convenience of AFLPs into a single assay (Vogel 1997) and have been applied to maize (Vogel, personal communication). Several carrot SSRs have been identified using Genbank and EMBL searches (Niemann et al. 1997); however, SAMPL markers have not been reported. The objectives of the present study were to construct a framework molecular map of carrot using RFLPs, RAPDs, AFLPs and the SAMPL procedure, and to map P_1, Y_2 and *Rs*.

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Materials and methods

Plant materials and DNA extraction

The mapping population of 103 F_2 plants was derived from a single F_1 plant of the cross between fertile maintainer inbreds B9304 and YC7262. B9304 (Simon et al. 1990) had non-purple root phloem, an orange xylem (core) and a high reducing sugar content ($p_1p_1y_2y_2$ *RsRs*). The roots of YC7262, a full-sib of B7262 (Simon et al. 1997), had purple phloem, a yellow xylem and a low reducing sugar content ($P_1P_1Y_2Y_2rsrs$). To ensure adequate tissue for DNA extraction, the F_2 plants were also propagated by tissue culture (Simon et al. 1990). Regenerated plants and plants from seed were grown in pots in the greenhouse. Young leaves from 50 to 90 day old plants were harvested, frozen in liquid nitrogen and lyophilized. Total DNA was isolated by a modified CTAB extraction method (Murray and Thompson 1980).

Phenotypic evaluations of P_1 and Y_2 were as described by Simon (1996). Briefly, roots with any visible purple pigmentation in an examination of phloem cross-sections were categorized as $P_1/\_$. Others were categorized as p_1p_1. Roots with a yellow core were categorized as $Y_2/\_$; those with an orange core as y_2y_2. Categorization of *Rs* involved placing root juice samples on filter paper, followed by exposure to dinitrosalicylic acid and heat as described by Simon and Freeman (1985).

Southern hybridization, genomic DNA library, and RFLP analysis

The procedures followed were as described by Vivek and Simon (1998) and are listed here briefly. DNA samples from a portion of the mapping population was singly digested with *Eco*RI and *Hin*dIII restriction enzymes according to the manufacturer's recommendations (Promega, Madison, Wis., USA). Electrophoresis of the digested DNA was in 0.8% agarose gels in 1 × TBE at 9 mA for 18 h. The DNA was blotted overnight to Zetaprobe (BioRad, Richmond, Calif., USA) filters by capillary transfer (Southern 1975).

A *Pst*I genomic library was cloned into pGEM 3Zf(−) from digested size-fractionated genomic DNA of the carrot cultivar 'Savory'. Four-hundred-and-forty white colonies on X-gal plates (Messing 1983) were picked from the library, individually grown overnight in LB broth with antibiotic, and plasmids isolated (Riggs and McLachlan 1986). Potentially single to low-copy number inserts were identified by the dot-blot procedure of Landry and Michelmore (1985). Probes were prepared as described by Sambrook et al. (1989) with modifications. Forty four (10%) of these clones gave a strong signal when hybridized with sheared genomic DNA. These were assumed to be repetitive and were eliminated from further analysis. The remaining 396 clones were assumed to be low-copy clones. Plasmids containing low-copy clones were digested with *Pst*I and electrophoresed on an agarose gel. A total of 300 inserts ranged from 500 bp to 2000 bp and were used for further analysis.

RAPD analysis

The primers evaluated were 164 randomly selected 10-mers from Operon Technologies, Alameda, Calif. (numbers 1–20 of series I, J, K, L, P, Q, and S, except for K11 and L10; N7–N17, and O4–O20). Parental DNAs were used for the initial screening of primers. Each primer was replicated at least twice.

All PCR reactions were performed on a Perkin Elmer 9600 thermocycler in a 15-µl vol containing 12 ng of carrot genomic DNA, 1 µM of primer, 2 mM $MgCl_2$, 100 µM of each dNTP, 1 × PCR buffer [50 mM KCl, 10 mM Tris-HCl pH (9.0), 10 mM NaCl, 0.1% Triton X-100], and 0.5 units of *Taq* DNA polymerase from Promega (Madison, Wis, USA).

The following cycling profile was employed: (1) initial denaturation at 94°C for 4 min; (2) three cycles at 94°C/15 s, 35°C/15 s, with a 59-s ramp to 72°C/75 s; (3) 40 cycles at 94°C/15 s, 40°C/15 s, with a 59-s ramp to 72°C/75 s; (4) a 72°C/10-min final extension and soak at 4°C. Amplification products were separated by electrophoresis in 1.6% agarose (Biorad) gels with 1 × TAE for 3 h at 100 V, stained with ethidium bromide and photographed under UV (Eagle Eye, Stratagene, La Jolla, Calif., USA). Potentially codominant RAPDs were run on 3% MetaPhor™ (high-resolution agarose, FMC Bioproducts, Rockland, Me., USA) in 1 × TBE for 3 h.

AFLP analysis

The procedures followed were according to the manufacturer's (GIBCO-BRL, Life Technologies, Gaithersburg, Md., USA) recommendations with slight modifications. DNA restriction digestion, ligation, and selective amplification reactions were performed at 1/4 the recommended volume and the pre-selective amplification was performed at 1/10 the recommended volume.

Seven AFLP primer combinations were evaluated (*Eco*RI-*Mse*I combinations numbered P1–P7, see Table 1). Polymorphic bands were visually identified and serially numbered from B1 up to B49, beginning with the largest fragment. The migration of each band was measured and the size, in base pairs, was estimated. Presence or absence of bands for each F_2 individual was scored as required by Mapmaker, version 2.0 (Lander et al. 1987).

SAMPL analysis

Reactions were done in the same way as the AFLPs except that SAMPL primers were used in place of the *Eco*RI primers. SAMPL primers were designed based on SSR knowledge from maize and soybean (Zietkiewicz et al. 1994). The primers employed were $ADB(CA)_6$, $HVH(AGC)_4$, $A(CA)_7(TA)_2T$, $A(GA)_7(TA)_2$, and $VHV(CT)_8$ (A – Adenine; T – Thymine; G – Guanine; C – Cytosine. The degenerate nucleotides were: B – no A; D – no C; H – no G; V – no T) (see Table 2). SAMPL primers were labelled and used in combination with unlabelled *Mse*I primers (M) having +2 (CT) and +1(C) extensions as selective nucleotides at the 3′ end (see Table 2).

Molecular-marker evaluation

Preliminary screening for each molecular marker was done using parental DNA and/or DNA from a subset of F_2s. As the individual parental plants were unavailable, DNA samples from bulked plants of the B9304 and B7262 populations were used as parental representatives for all molecular-marker evaluations.

Data scoring, linkage analysis, and map construction

The symbols required by Mapmaker version 2.0 (for the MacIntosh) were used to score the bands (Lander et al. 1987). Where the parental allelic phase was unknown, markers were double-scored and the allele origin deduced by association with phase-known linkages (Gomez et al. 1996).

All markers were entered into Mapmaker MacIntosh version 2.0 (Lander et al. 1987) for analysis. Markers were grouped with the two-point "group" command at LOD = 4.0 and a maximum recombination (θ) threshold of 0.25. The groups were mapped using the Kosambi (1944) mapping function.

Results

RFLP analysis

A total of 84 (28%) of the 300 clones evaluated for parental DNAs (B9304 and B7262) had clear scorable bands with little or no background signal. Thirty (36%) of these scorable 84 clones showed polymorphisms after *Hin*dIII digestion in the F_2 population whereas the remaining 54 were not polymorphic with this enzyme.

RAPD analysis

Amplified products of 164 primers were evaluated. Of these, 33 (20%) were polymorphic between the parents, but only two of these (S3-500 and K9C) could be scored without ambiguity and they were mapped. K9C was codominant.

AFLP analysis

Seven primer combinations amplified 404 bands of which 164 (42%) were polymorphic in the F_2 population (Table 1). There were five (3%) length-variant (codominant) markers. Among primer pairs there was an approximately two-fold range in the number of bands (36–75), polymorphic bands (19–31), and % polymorphism (31–53). Only 123 bands which could be scored without ambiguity across all the F_2s were used for mapping.

SAMPL analysis

Five SAMPL primers (three simple: S1, S2, S5; and two compound: S3, S4), were evaluated in combination with an *Mse*I primer having +1 (C) and +2 (CT) extensions for a total of ten primer combinations (Table 2). SAMPL4 did not generate any bands and SAMPL2/ MCT generated unclear bands. Of the seven remaining primer combinations, only SAMPL3/MCT was evaluated on all the F_2s. More than 100 bands were observed. Two pairs of bands which differed in size by only a few base pairs, segregated as codominant markers (one or both of the presumed allelic bands of each marker was present in each F_2) for the SAMPL3/ MCT combination (markers Smp3C2 and Smp3C3, Fig. 1). These markers were scored and mapped. Other SAMPL mapping was not attempted.

Segregation analysis

A total of three phenotypic markers, eight RFLPs, two RAPDs, two SAMPL markers and 123 AFLPs were scored (Tables 3 and 4). Since all the F_2 plants were derived from a single F_1 plant the χ^2 goodness-of-fit tests were performed for expected F_2 ratios of 3 : 1 for dominant and 1 : 2 : 1 for codominant markers ($\alpha = 0.05$

Table 2 List of SAMPL and *Mse*I primers used and the number of bands seen with each combination evaluated on 103 F_2 plants

SAMPL primer	Sequence[a]	*Mse*I primer[b]	
		MC	MCT
S1	$ADB(CA)_6$	13	6
S2	$HVH(AGC)_4$	>100	Unclear bands
S3	$A(CA)_7(TA)_2T$	>100	>100[c]
S4	$A(GA)_7(TA)_2$	No amplification	No amplification
S5	$VHV(CT)_8$	10	8

[a] A – Adenine, T – Thymine, G – Guanine, C – Cytosine; degenerate nucleotides: B – no A, V – no T, H – no G, D – no C
[b] M = *Mse*I primer with C (Cytosine) and T (Thymine) as selective nucleotides at the 3′ end
[c] Includes the only two mapped SAMPL markers (Smp3C2 and Smp3C3)

Table 1 AFLP primer combinations evaluated and the banding patterns revealed among 103 F_2 plants

Primer no.	Primer combination[a]	No. of bands	Polymorphic bands	% Polymorphic	Codominant markers	% codominant markers	No. mapped
P1	EAAC/MCAA	62	26	42	0	0	18
P2	EAAC/MCTA	56	24	43	0	0	15
P3	EAAC/MCAT	62	23	37	0	0	16
P4	EAAG/MCTT	75	31	41	2	6.5	16
P5	EACT/MCTG	36	19	53	0	0	12
P6	EAAG/MCAA	68	21	31	2	9.5	10
P7	EACA/MCAG	45	20	44	1	5	9
Total		404	164	–	5	–	96
Average		58	23	42	0.7	3	14

[a] E – *Eco*RI primer, M – *Mse*I primer

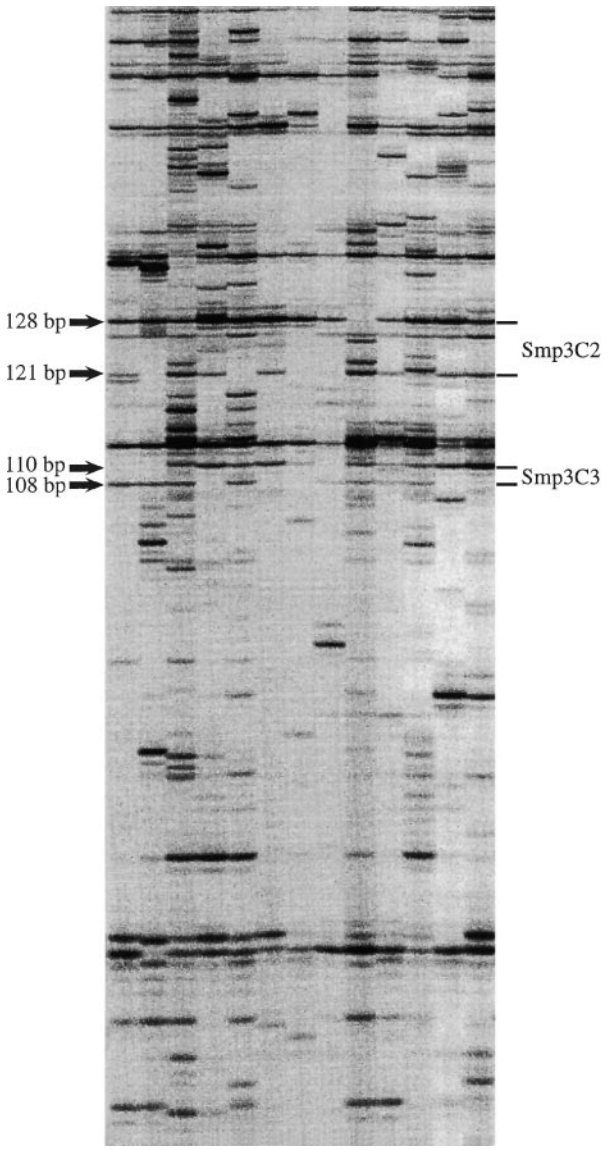

Fig. 1 SAMPL variation in carrot for markers Smp3C2 (allelic bands of 121 bp and 128 bp), which maps to linkage group D, and Smp3C3 (allelic bands of 108 and 110 bp), which maps to linkage group C. Each lane represents a different F_2 plant

and 0.01 levels, data not presented). None of the phenotypic markers deviated significantly from the expected ratio of 3:1 at the 1% level. P_1 and *Rs* exhibited disturbed segregation at the 5% level. Two (25%) of eight RFLPs were disturbed at 5% and one (13%) at 1%. All the RAPD and SAMPL markers conformed to expected ratios of either 3:1 (for dominant) or 1:2:1 (for codominant) markers. Twenty one (17%) and ten (8%) of 123 AFLPs deviated from expected ratios at 5% and 1%, respectively. Overall, 25 markers (18%) out of 138 scored exhibited disturbed segregation at 5%, and ten markers (7%) were disturbed at 1%.

Table 3 Markers evaluated for carrot genetic mapping

Marker type	Markers scored	Number linked	Number codominant	Unlinked markers
Phenotypic loci	3	3	0	0
RFLPs	8	6	8	2
RAPDs	2	2	1	0
AFLPs	123	96	1	27
SAMPL	2	2	2	0
Total	138	109	12	29

Linkage analysis

At LOD = 4.0 and a maximum recombination value (θ) = 0.25, 11 linkage groups of five or more markers (A through K) were obtained (Table 4, Fig. 2). Of the 29 markers not included in these linkage groups, there were seven groups with two markers each, one group with three markers, and 12 unlinked markers (data not shown).

Molecular markers linked to the phenotypic traits analyzed are of particular interest. The AFLP markers P6B15 and P2B12XA flanked P_1 by 1.7 cM and 8.1 cM, respectively. P_1 and P6B15 exhibited disturbed segregation at the 5% level. These were the only skewed markers on linkage group A. AFLP P1B34 was 2.2 cM from Y_2 on linkage group B. *Rs* exhibited disturbed segregation and mapped to the end of linkage group C with AFLP P3B30XA, 8.1 cM away and not disturbed.

General map coverage

RAPDs, SAMPL markers, and phenotypic loci each represented 2% to 3% of the linked markers. About 6% of the markers were RFLPs with the remaining 88% being AFLPs. The map had a total of 10 (9%) codominant markers out of the 109 linked markers. The total map length was 534.4 cM with an average marker-to-marker distance of 4.9 cM. Of the 99 dominant linked markers, 63 (64%) of them had the B9304 null-allele (absence of band) while the remaining 36 (36%) had the null-allele from YC7262.

Discussion

Identification of molecular markers

RFLP variation has been examined in several crops (reviewed in Altenbach 1995). In the present study 10% of the clones in the library were estimated to be high copy. Schulz et al. (1994) found 25% of the clones to be high copy. Sampling differences could account for this variation.

Table 4 Marker distribution on linkage groups

Groups	Number of markers								Length (cM)	Spacing (cM)
	Phenotypic	RFLP	RAPD	AFLP	SAMPL	Total	Codominant	Skewed		
A	1	2	0	13	0	16	2	2	89.4	5.6
B	1	1	1	5	0	8	1	0	61.6	7.7
C	1	0	0	10	1	12	1	2	103.9	8.7
D	0	1	0	7	1	9	2	0	36.5	4.1
E	0	1	0	11	0	12	2	3	62.8	5.2
F	0	1	0	11	0	12	1	0	33.7	2.8
G	0	0	0	9	0	9	0	3	35.1	3.9
H	0	0	1	10	0	11	1	3	50.3	4.6
I	0	0	0	5	0	5	0	1	21.6	4.3
J	0	0	0	7	0	7	0	0	13.0	1.9
K	0	0	0	8	0	8	0	1	26.6	3.3
Total	3	6	2	96	2	109	10	15	534.4	

Average # markers per group = 9.91
Average distance between markers = 4.9 cM
Average length/group = 48.6 cM

The percentage of clones detecting polymorphisms found in this study (28%) is comparable to that of Schulz et al. (1994). *Hin*dIII detected the most polymorphism in our case whereas Schulz et al. (1994) reported that *Xba*I and *Hin*dIII detected similar levels of polymorphism. Similarly, 20% of the RAPD primers detected polymorphisms in the parental DNAs in our study and also in the mapping population of Schulz et al. (1994).

This is the first report of AFLPs in Apiaceae (Umbelliferae). An average 42% of all bands were polymorphic, which is 2.5-times more variation than that detected by RFLP probes and double that of RAPD primers. Variation in the number of bands obtained from each SAMPL primer combination is an indication of the prevalance of the particular SSR. The simple repeat (AGC) (primer S2) appeared to be more abundant than (CA) or (CT) (primers S1 and S5, respectively) in carrot. The occurrence of the compound repeat motif $(CA)_7(TA)_2$ (primer S2) was widespread in contrast to the compound repeat $A(GA)_7(TA)_2$ (primer S4) which was absent in carrot.

Segregation and linkage analysis

Using a stringent LOD score of 4.0 and $\theta = 0.25$ a conservative 109-point linkage map consisting of 11 linkage groups was obtained, rather than the nine groups expected. This covered 534.4 cM of the genome with a marker spacing of 4.9 cM. Clearly the map is unsaturated. The addition of more markers may establish fewer groups. By comparison, the four carrot maps obtained by Schulz et al. (1994) had 5–8 linkage groups with 19–26 markers and covered a map length of 800 cM with a spacing of 13.1 cM.

The overall rate of disturbed segregation in this study, 18%, is comparable to the 24% found by Schulz et al. (1994). Disturbed segregation of the AFLP data could arise from PCR-amplification inefficiencies, whereas phenotypic traits like P_1 demonstrate disturbed segregation due to incomplete penetrance (Simon 1996). Both marker classes can also be disturbed by meiotic perturbations. The coincidental disturbed segregation of both P_1 and its mostly linked molecular marker, P6B15, is more readily explained by meiotic disturbance. The diversity of the U.S. and Turkish parents in our study could have contributed to meiotic irregularities. Disturbed segregation was reported in interspecific crosses of tomato (Nienhuis et al. 1987). Zenkteler (1962) observed carrot pollen abortion associated with meiotic chromosome-ring formation. Chromosome pairing in the material of the present study has not yet been investigated.

All the RAPD and SAMPL markers were linked to other markers, while 27 (22%) of 123 scored AFLPs were unlinked. This could have been due to inefficiency generally associated with dominant markers, like AFLPs, to detect linkages in the F_2s, poor amplification of AFLP bands, or incomplete marker saturation of the genome. Unlinked RFLPs were also observed and could have been due to incomplete saturation of the linkage groups or missing data from some members of the mapping population.

The inheritance studies of Simon (1996) showed that P_1, Y_2 and *Rs* were unlinked. Our molecular genetic linkage study is consistent with that result. Zenkteler (1962) analyzed the meiotic chromosomes of carrot and found one chiasma per chromosome arm. With a total of 18 chiasmata, each translating to a 50-cM genetic distance, the carrot genome was estimated to be 900 cM in length. The total map length found in this

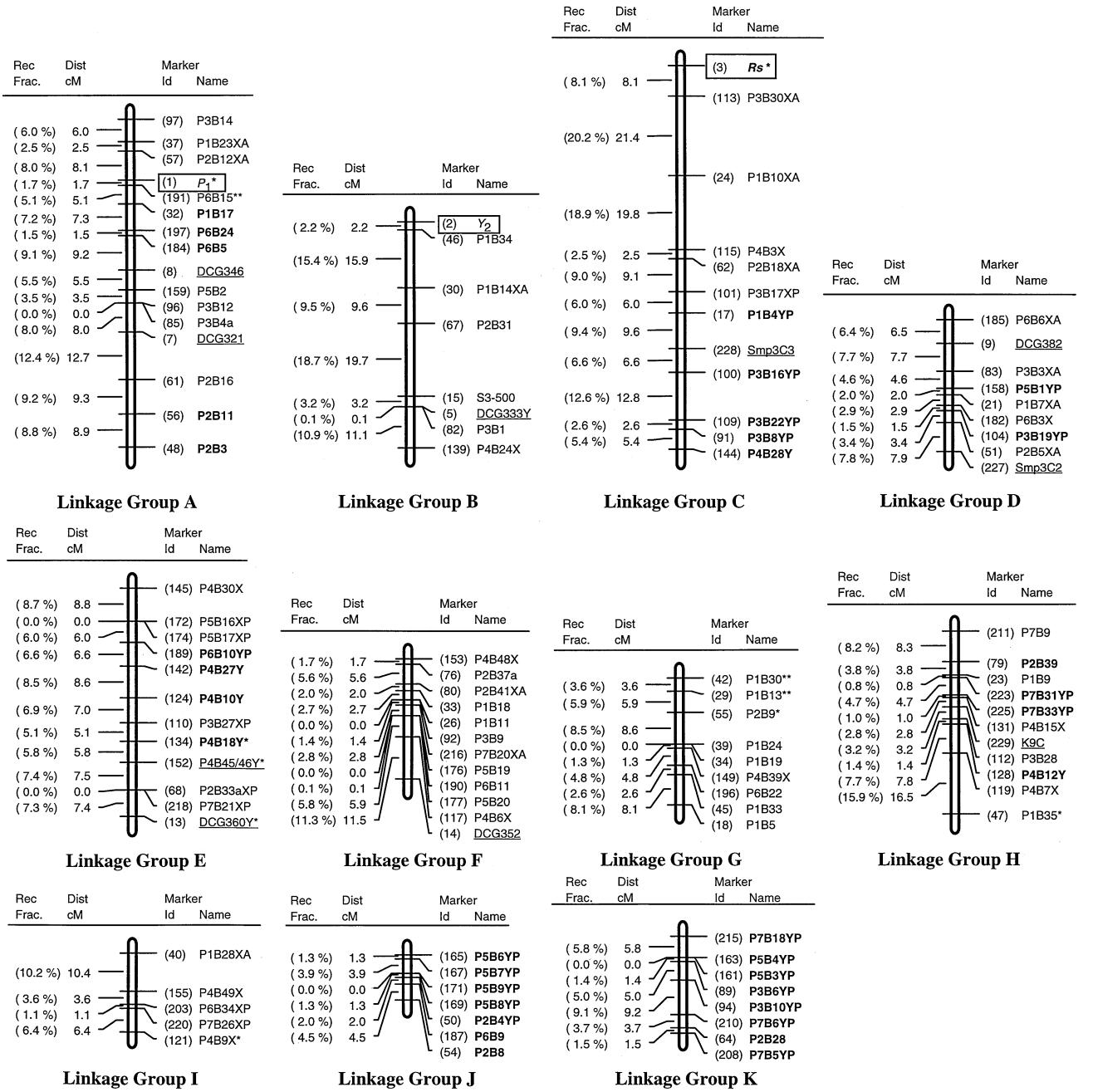

Fig. 2 Linkage groups A through K of the carrot genetic map. Markers mapped at LOD = 4.0 and $\theta = 0.25$. All the linkage groups have been drawn to scale with 1-cm length = 10-cM genetic distance. RFLPs – marker names starting with "DCG"; RAPDs – markers K9 and S3-500; AFLPs – marker names starting with "P"; SAMPL – marker names starting with "Smp". *Underline* – codominant marker; *bold letters and/or suffix Y* – null allele is from YC7262; *regular letters and/or suffix X* – null allele is from B9304. * – skewed at $\alpha = 0.05$, ** – skewed at $\alpha = 0.01$

study was 534.4 cM. An expansion of map length is expected as more markers are added.

About 88% of the markers on the map consisted of AFLPs, 3% were phenotypic loci, 6% RFLPs, 2% RAPDs and 2% SAMPL. Of the 109 mapped markers, 99 (91%) were dominant while ten (9%) of the markers were codominant. Only codominant SAMPL were scored. All RFLPs and one of two RAPDs were codominant. About 99% of AFLPs scored for mapping were dominant while the remaining 1% were codominant. In an F_2 population the relative efficiency of dominant markers in detecting recombination fractions is at best half of that of codominant markers when there is tight coupling-phase linkage between any two markers (Mather 1951; Allard 1956; Reiter et al. 1992) and this efficiency decreases as the distance between them increases. Repulsion phase-linked dominant markers are most efficient at 50% recombination

(no linkage) and their efficiency decreases as the markers approach tight linkage. To improve the power of this carrot mapping effort one could: (1) use more codominant markers; (2) use dominant markers with recombinant inbred lines, doubled haploids, or backcross progeny; (3) use two dominant markers closely linked in repulsion (i.e., markers amplified from different parents) as a "codominant locus" (Williams et al. 1993); or (4) convert the AFLPs scored as dominants to codominants by either scoring the autoradiograms for intensity polymorphism or converting AFLP bands to codominant PCR-based markers using inverse PCR (Bradeen and Simon 1998).

The map we developed forms a strong foundation for mapping other traits in carrot, including QTLs. Fine mapping of P_1, Y_2, and a nematode resistance locus are underway.

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