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V. Laucou · K. Haurogné · N. Ellis · C. Rameau

Genetic mapping in pea. 1. RAPD-based genetic linkage map of *Pisum sativum*

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Abstract A genetic linkage map of *Pisum sativum* L. was constructed based primarily on RAPD markers that were carefully selected for their reproducibility and scored in a population of 139 recombinant inbred lines (RILs). The mapping population was derived from a cross between a protein-rich dry-seed cultivar 'Térèse' and an increased branching mutant (K586) obtained from the pea cultivar 'Torsdag'. The map currently comprises nine linkage groups with two groups comprising only 6 markers ($n = 7$ in pea) and covers 1139 cM. This RAPD-based map has been aligned with the map based on the (JI281 × JI399) RILs population that currently includes 355 markers in seven linkage groups covering 1881 cM. The difference in map lengths is discussed. For this alignment 7 RFLPs, 23 RAPD markers, the morphological marker *le* and the PCR marker corresponding to the gene *Uni* were used as common markers and scored in both populations.

Key words Linkage mapping · *Pisum sativum* · Random amplified polymorphic DNA · Recombinant inbred lines

Introduction

Since the development of the polymerase chain reaction (PCR) in generating random amplified polymorphic DNA (RAPD) in 1990 (Welsh and McClelland 1990; Williams et al. 1990), this technique has proven valuable in the construction of genetic maps in several species and in the production of genetic markers linked to specific phenotypic traits in particular using bulked segregant pools (Michelmore et al. 1991). RAPD technology became popular because of its simplicity and ease of use in a modestly equipped laboratory in contrast to restriction fragment length polymorphism (RFLP) technology which is time-consuming and labor-intensive.

Since then, other PCR-based techniques have been described. In particular, amplified fragment length polymorphism (AFLP™) was developed (Vos et al. 1995) as a new DNA marker system combining the features of RFLP and PCR. This technique has rapidly proven to be efficient in generating a large number of genetic markers in a single experiment (high multiplex ratio) with a high reproducibility (Powell et al. 1996; Jones et al. 1997). AFLP, which is less easy to use than RAPD, is particularly adapted for constructing high-density maps and for the positional cloning of genes of interest (Thomas et al. 1995).

In the present paper, we describe the construction of a pea genetic map based primarily on RAPD markers that were carefully selected for their reproducibility between different experiments presented here and in an accompanying paper (Rameau et al. 1998). Only intensely staining and clearly resolvable bands were used and mapped. The main objective of the development of this map is to constitute a framework for genetic studies in particular for localizing mutations, genes and quantitative trait loci (QTLs) controlling plant architecture using PCR markers. The construction of the present linkage map used a population of recombinant inbred lines (RILs) derived from the cross 'Térèse' × K586. 'Térèse' is a dwarf (*le*) protein-rich, dry-seed cultivar that we used in a mutagenesis program where the M2 plants were screened for altered apical dominance (Rameau et al. 1997). K586 is a branching

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V. Laucou · K. Haurogné · C. Rameau (✉)
Station de Génétique et d'Amélioration des Plantes,
Institut National de la Recherche Agronomique,
Route de Saint-Cyr, 78026 Versailles Cedex, France
Fax : +3-1-30 83 33 19
E-mail: rameau@versailles.inra.fr

N. Ellis
Department of Applied Genetics, John Innes Centre, Colney Lane,
Norwich NR4 7UH, UK

mutant derived from the WT tall (*Le*) cultivar 'Torsdag' (Arumingtyas et al. 1992). 'Torsdag' is the genotype from which several mutants were obtained by Dr K.K. Sidorova (Institute of Cytology and Genetics, Novosibirsk, Russia), and it is used in many physiological mutant-based experiments (Reid and Ross 1993; Murfet and Reid 1993). The choice of these parents lies in the fact that several mutations are available in the 'Térèse' and 'Torsdag' backgrounds. Further mapping of various mutations will take advantage of this choice: for example, a mutation on the 'Torsdag' background can be mapped using the F_2 population derived from the cross of the mutant line with 'Térèse'. We have two of the advantages derived from not working with several different crosses; namely, (1) polymorphic RAPD markers in this cross are known, and (2) linkage relationships between these markers are not confused by possible translocation events. In an accompanying paper, genes with a major effect on plant architecture, and in particular on branching, have been mapped using such isogenic lines (Rameau et al. 1998).

Because the application of molecular markers for genetic studies and plant breeding relies on the ability to collate information from genetic maps obtained from different crosses, this RAPD-based map has been aligned with the RFLP map from Ellis et al. (Ellis et al. 1992) of the population (JI281 × JI399) RILs. This map has been recently updated and revised in the light of cytogenetic data (Hall et al. 1997a, b) and enriched with anchored PCR markers corresponding to MADS-box genes and *Ty*1-*copia*-like insertions (Ellis et al. 1998). For this alignment, some RFLP but mainly RAPD markers were used as common markers. The results show that if carefully selected, RAPD markers can be transferred from one cross to another.

Materials and methods

Plant material

A total of 200 F_2 plants from the cross 'Térèse' × K586 were self-pollinated and advanced to the F_7 generation using single-seed descent. The final population used for DNA extraction consisted of 139 lines. RILs from the cross JI281 (*Pisum sativum* from Ethiopia) × JI399 (= cv 'Cennia') were obtained from the John Innes *Pisum* Germplasm collection. The number of lines analyzed from this cross was 71.

Markers and probes scored in the population ('Térèse' × K586) RILs

The pre-screening of RAPD fragments suitable for mapping was performed with the two parents 'Térèse' and 'Torsdag' (isogenic wild-type of the mutant line K586) and on 46 F_2 plants from the cross 'Térèse' × 'Torsdag'. Moreover, some RAPD markers not selected during this screening (because the intensity of the band was slightly too faint) but which were found linked to a gene of interest during the bulk segregant analyses described in Rameau et al. (1998) were also analyzed on the population of RILs when possible.

Other molecular markers which have been mapped in previous studies were also analyzed: two RFLPs mapped in Dirlewanger et al. (1994), ED156 and ED252, which mapped at 15.9 from *mo*, a gene conferring resistance to the pea common mosaic virus, and seven RFLP probes used in Ellis et al. (1992). The SCAR $PD10_{650}$, which is closely linked to the gene *er-1* conferring resistance to powdery mildew (Timmerman et al. 1994) on linkage group VI, was not polymorphic between 'Térèse' and 'Torsdag', but the RAPD marker, $NW04_{950}$, linked to *sbm-1* on the same linkage group (Timmerman et al. 1993) has been mapped.

Mapping of the PCR marker *UNI*

The pea cDNA homolog of the floral meristem identity genes *FLORICAULA* in *Antirrhinum* and *LEAFY* in *Arabidopsis* and corresponding to the gene *Uni* in pea (Hofer et al. 1997) has been transformed to a PCR marker. Two primers flanking the first intron generated a 536-bp product from 'Térèse' and 'Torsdag': the forward primer (5′AACGCTCTCGATGCTCTC3′) at the end of the first exon and the reverse primer (5′CCAGGCTCCGTCACAATGAA3′) in the middle of the second exon. The PCR conditions were 35 cycles at 94°C, 60 s; 52°C, 60 s; 72°C, 2 min. These products were cloned using T-vectors (Hengen 1995), and their sequences were compared to find a polymorphic restriction site. A restriction site with *Bsm*I was found in the intron for 'Térèse' but not in 'Torsdag'. A migration of 5 h in an agarose gel of 3% enables the profiles of 'Térèse' and 'Torsdag' to be distinguished easily.

DNA extraction

Genomic DNA was isolated from leaf tissue (stipule and/or leaflet) from individual F_7 plants. The tissue was immediately frozen in liquid N_2 and stored at -80°C. DNA was isolated using a modified version of the protocol described in Doyle and Doyle (1987). For the RAPD technique, approximately 0.2 g of the frozen samples was ground to a powder in liquid N_2 using a chilled mortar and pestle and then transferred to sterile 1.5-ml Eppendorf tubes. Buffer (800 µl; 100 m*M* TRIS-HCl pH 7.5, 0.7 *M* NaCl, 10 m*M* EDTA, 1% CTAB, 1% β-mercaptoethanol) was added to each tube prior to incubation at 60°C for 30 min. Subsequently, 400 µl of chloroform/1-octanol (24 : 1) was then added to the tubes, and the contents were mixed by gentle inversion. The samples were centrifuged at 4500 rpm for 10 min and the upper aqueous phase was transferred to new 1.5-ml tubes with 700 µl of propanol-2. After centrifugation at 4500 rpm for 10 min, 2 µl of 5 *M* ammonium acetate was added, followed by 1 ml of 70% ethanol. The precipitated DNA was washed twice in 70% ethanol, air-dried and resuspended in 100 µl of TE buffer.

The same protocol of DNA extraction was used for RFLPs but starting with about 3 g of fresh tissue. For this quantity of tissue, 11 ml of preheated extraction buffer was added to the tissue ground to powder in a 30-ml tube.

RAPD procedure

Genomic DNA was used as a template for PCR amplification. A single 10-mer oligonucleotide primer with an arbitrary sequence (Operon Technologies, Alameda, Calif.) was used in each PCR amplification. Amplification was carried out, using a method similar to that described in Welsh and McClelland (1990), in 25 µl of a solution that contained 10 m*M* TRIS-HCl pH 9.0, 50 m*M* KCl, 1.5 m*M* $MgCl_2$, 0.1% Triton-X-100, 0.2 mg/ml BSA, 100 µ*M* of each dNTP, 20 ng of 10-mer primer, 10 ng of genomic DNA and 0.5 unit *Taq* DNA polymerase (Appligene Oncor, France). The amplifications were performed in a Perkin Elmer Cetus 480 thermal cycler

(Norwalk, Conn.) or a PTC 100 thermocycler (MJ Research, Watertown, Mass.) for 45 cycles, after a denaturation at 94°C for 5 min. Each cycle consisted of 1 min at 94°C followed by 1 min at 40° or 45°C (generally) and 2 min at 72°C. The amplification products were separated by electrophoresis (4 V/cm) through a 1.4% agarose gel, stained with ethidium bromide and viewed and photographed under ultra-violet light.

RAPD marker naming

Each RAPD marker was given a two-part name. The first part corresponded to the primer with which the polymorphism was observed (one or two letters followed by a two-digit number which corresponds to an Operon Technologies primer), while the second part corresponded to the approximate size (in bp) of the band.

The RFLP technique

For Southern blot analysis, 7–8 μg of genomic DNA was digested with 30–40 units of *Eco*RI or *Hin*dIII in 50 μl for 4–6 h at 37°C and electrophoresed through a 0.7% agarose, 1 × TAE gel. The gel dimensions were 150 × 250 mm and the total volume of agarose was 200 or 250 ml. Voltage gradients were usually 21 V for 24 h, followed by 25 V for 5 h. Following electrophoresis, the gel was soaked in 0.25 *N* HCl for 15 min, then in 0.4 *N* NaOH for 20 min. The gel was blotted to a charged nylon membrane by capillary transfer in 0.4 *N* NaOH. After blotting overnight, the nylon membranes were rinsed in 2 × SSC.

Prehybridization, hybridization and washing conditions were adapted from Church and Gilbert (1984). Blots were prehybridized for 2–4 h at 65°C in a buffer containing 7% SDS, 1 m*M* EDTA, 0.5 *M* Napi (we used only Na_2HPO_4 instead of a mix of Na_2HPO_4/ NaH_2PO_4). Hybridizations were carried out at 65°C overnight in the same buffer with the addition of 30–50 ng of ^{32}P probe DNA. The membranes were generally washed three times (for 20 min, 15 min, 15 min) in a buffer containing 1% SDS, 40 m*M* Napi, at 65°C. Autoradiography was performed by exposing the membranes to films at − 80°C with intensifying screens. The probes used are listed in Table 1 and are described in Dirlewanger et al. (1994) and Ellis et al. (1992).

Linkage analysis

Each marker was tested for a 1:1 segregation ratio using Chi-square tests. All markers with a Chi-square value greater than 3.84 ($\alpha = 0.05$) were excluded from the linkage analysis except for those markers used as common markers. Their approximate position was given even if they showed a distorted segregation in one population. Segregation data were analyzed with the MAPMAKER/EXP 3.0 computer program (Lander et al. 1987; Lincoln et al. 1992). The Haldane function was used to obtain the centiMorgan (cM) values. Markers were assigned to linkage groups using the "group" command with a LOD $\geq$ 5.0 or $\geq$ 4.0 and a map distance below 30 cM.

Within each group, three-point linkage analyses were conducted on a subset of markers found by the "suggest subset" command, which selects informative well-spaced markers. The most likely order of markers within this subset was assessed with the "order" command. The remaining loci in each group were then placed with the "try" command if a position at least 50 times more likely than the next most likely position could be found. Loci that had been excluded on the basis of the three-point linkage data were given an approximate position using the "near" command. The loci whose positions were ambiguous were noted. Finally, the likelihoods of the ordered linkage groups were tested against the likelihoods of all maps obtainable by permuting the orders of all adjacent triplets ("ripple" command).

Table 1 List of the RFLP markers scored in population ('Térèse' × K586) RILs

Marker	TE[a]	TO[a]	Chi[b]
cDNA 136	28	49	5.7
cDNA 187	74	60	1.5
cDNA 228	72	58	1.5
cDNA 324	58	61	0.1
pCHS1	53	53	0.0
Vc5	58	76	2.4
pSTL	72	63	0.6
ED 156	64	54	0.9
ED 252	74	62	1.1

[a] Number of RILs with the profile of 'Térèse' (TE) or 'Torsdag' (TO)
[b] Chi-square value of goodness-of-fit to 1:1 ratio

Results

Screening of RAPD markers for the map of population (Térèse × K586) RILs

A total of 620 primers (kit A–AE) were pre-screened against the parents 'Térèse' and 'Torsdag'. Primers which gave polymorphism were also tested on 46 F_2 plants, obtained from the cross 'Térèse' × 'Torsdag' (data not shown). Well-defined polymorphic bands were selected if they were consistently and readily observed. For each primer, an annealing temperature was chosen which optimized the amplification of the polymorphic band of interest and the ease of reading the gel (Table 2). The annealing temperature was generally 40° or 45°C, although an annealing temperature of 36° or 48°C was preferable for a few primers. Only 157 of the 620 primers initially screened were selected. A strong selection of polymorphic fragments was performed to avoid the well-known problem of RAPD data reproducibility (Ellsworth et al. 1993; Jones et al. 1997). The selected primers amplified a total of 247 putatively segregating polymorphisms, 133 from 'Térèse' and 114 from K586 (Table 2). Fragment sizes ranged from about 100 bp to 3000 bp. Chi-square values for goodness-of-fit to a 1:1 Mendelian ratio of the 249 markers scored (two morphological and 247 RAPD) are indicated in Table 2. Of the 249 loci 20 exhibited a distorted segregation and were excluded from the analyses.

Two-point analysis

Two-point analysis has been done on 240 markers: two morphological (*le*, *af*), 228 RAPDs [227 screened above and NW04 from Timmerman et al. (1993)], the PCR marker UNI and nine RFLPs which were found polymorphic in our cross [two already mapped in

Table 2 List of the 249 markers (two morphological and 247 RAPD markers) scored in population ('Térèse' × K586) RILs

Marker	TE[a]	TO[a]	Tann[b]	Chi^{2c}
af		+		0.18
le		+		0.01
A04-1500		+	45	0.18
A04-800		+	45	0.18
A04-430	+		45	0.35
A06-1280	+		45	1.42
A08-1250		+	40	0.06
A11-900		+	40	0.06
A14-680		+	40	0.18
A19-870		+	40	0.06
A19-630		+	40	2.08
B01-1350	+		40	0.06
B01-960	+		40	0.01
B07-1750	+		40	0.06
B07-620		+	40	15.9**
B11-1700		+	45	0.01
B12-1200	+		48	0.87
B19-1900		+	40	0.06
B19-600	+		40	0.58
B20-300		+	40	0.87
C01-730		+	45	2.11
C01-580		+	45	0.87
C07-2000		+	45	0.18
C07-1300		+	45	0.35
C07-740		+	45	0.26
C10-1150		+	40	0.35
C12-650	+		45	0.00
C12-500	+		45	1.04
C14-1470	+		45	0.35
D03-1200	+		40	1.62
D03-1100		+	40	0.87
E01-1380	+		40	0.03
E01-870		+	40	0.87
E08-980		+	36	1.22
E08-600	+		36	1.22
E09-700		+	40	0.26
E11-900		+	40	0.87
E11-600	+		40	0.01
E12-800		+	40	1.22
E12-490		+	40	1.62
E13-1010	+		45	0.06
E16-1630	+		40	0.01
E16-650		+	40	2.08
E16-550		+	40	0.58
F07-2040		+	40	0.72
F08-1700		+	40	0.18
F09-1300	+		45	0.18
F20-1650	+		40	1.25
F20-980		+	40	0.01
G04-2000	+		40	2.08
G04-950		+	40	0.01
G04-500		+	40	0.35
G09-650		+	40	0.46
G10-1800		+	40	1.04
G10-1000	+		40	0.36
G10-980	+		40	1.64
G11-410		+	40	0.18
G12-650		+	45	1.62
G16-1200		+	45	67.7**
G16-750		+	45	2.11
G16-600	+		45	0.01
H01-1000	+		45	3.92*
H05-530	+		45	0.35
H11-1640	+		45	0.01
H11-600	+		45	0.26

Table 2 Continued

Marker	TE[a]	TO[a]	Tann[b]	Chi^{2c}
H14-1250		+	40	1.22
H14-620	+		40	0.06
I01-700		+	40	0.26
I10-1300	+		45	4.90*
I11-1020	+		45	0.06
I16-740	+		40	0.06
I19-900	+		40	8.81**
J04-900	+		40	1.62
J07-1100		+	40	6.05*
J11-1000	+		36	0.18
J11-500		+	36	0.87
J12-1280		+	40	0.87
J14-1500		+	40	0.58
J14-850		+	40	0.18
K02-800		+	40	1.22
K02-750	+		40	1.22
K02-600	+		40	62.2**
K03-3000		+	40	0.06
K03-1350		+	40	0.01
K03-600	+		40	1.22
K04-800	+		45	0.06
K08-2040	+		45	0.12
K16-900	+		40	0.35
K16-600	+		40	6.91**
L13-1350		+	40	0.06
L13-1180	+		40	0.06
L13-1000		+	40	6.91**
L13-920		+	40	0.01
L17-1050		+	40	0.35
L19-700	+		45	1.62
L19-550		+	45	0.06
M06-1350	+		48	0.72
M08-900	+		36	0.58
M16-1300	+		40	0.01
M17-1000	+		40	2.60
N01-720	+		45	0.03
N03-500	+		45	0.46
N11-1300		+	48	2.60
N11-700		+	48	0.58
N13-900	+		45	0.72
N13-600	+		45	1.62
N14-1050		+	45	0.35
N14-950	+		45	14.6**
N20-550		+	45	0.18
O01-700	+		40	0.36
O01-450	+		40	3.27
O04-1400	+		40	0.01
O07-1000	+		45	0.18
O09-1650	+		40	3.32
O09-800	+		40	2.08
O09-780		+	40	16.7**
O16-2500	+		45	9.85**
O16-950		+	45	1.86
O16-800	+		45	0.26
O18-1050		+	40	0.36
O18-800	+		40	1.04
O19-1200	+		40	0.06
O19-1050	+		40	0.35
P02-1000	+		45	0.36
P04-860		+	40	0.87
P05-1600	+		40	4.17*
P05-1000		+	40	0.72
P05-600	+		40	0.72
P05-100		+	40	0.47
P10-500	+		40	0.06
P11-600		+	40	0.35

Table 2 Continued

Marker	TE[a]	TO[a]	Tann[b]	Chi[2c]
P11-450		+	40	2.64
P16-2000		+	45	0.12
Q04-1050	+		40	24.1**
Q08-1000		+	40	0.03
Q08-950	+		40	0.00
Q09-550	+		40	0.35
Q17-740		+	40	0.87
Q20-950		+	40	0.87
R03-2000		+	40	2.60
R03-700		+	40	0.87
R04-900	+		40	0.01
R04-500	+		40	2.08
R05-450	+		40	0.07
R06-850	+		40	0.87
R06-650		+	40	0.58
R11-730	+		40	0.01
R12-320	+		45	0.01
R13-1550	+		45	1.04
R13-1200	+		45	0.26
R13-850		+	45	0.88
R17-550		+	40	1.48
R19-1100		+	40	0.88
S01-2000		+	40	0.06
S01-1800		+	40	2.08
S02-1400		+	36	0.36
S04-750		+	40	1.22
S09-1650	+		40	1.62
S13-3000	+		40	0.47
S18-650		+	40	4.50*
T02-840	+		40	0.06
T03-650	+		40	0.18
T11-800	+		40	0.00
T12-1000	+		45	0.87
T14-700		+	40	1.22
U01-1200		+	45	0.26
U06-900	+		45	3.17
U06-320	+		45	0.03
U07-1500		+	45	0.87
U08-650		+	45	0.88
U20-650		+	45	4.50*
V03-2400	+		40	0.60
V03-2000	+		40	0.03
V03-800	+		40	0.36
V06-800		+	45	0.01
V06-600	+		45	0.18
V07-650		+	45	1.62
V12-1600		+	40	1.62
V12-1150		+	40	0.06
V12-730		+	40	3.17
V16-1800	+		45	0.28
V16-1350		+	45	0.01
V16-900	+		45	0.46
V17-1400	+		45	0.03
V17-950		+	45	0.72
V18-450	+		45	0.87
V18-300	+		45	8.81**
V20-500	+		45	0.26
W05-280		+	40	0.12
W11-1200		+	45	1.62
W14-550	+		40	6.91**
W18-800	+		45	1.62
W19-900	+		40	0.35
X01-420	+		45	3.81
X02-450		+	45	0.87
X03-1600		+	45	0.46
X03-500		+	45	0.35

Table 2 Continued

Marker	TE[a]	TO[a]	Tann[b]	Chi[2c]
X03-400		+	45	0.18
X15-900	+		40	0.07
X16-1650	+		40	2.60
X16-1150		+	40	0.87
X17-1500	+		45	0.58
X17-500	+		45	0.18
X18-2200	+		40	0.01
Y02-1200		+	45	0.87
Y02-700		+	45	0.06
Y13-980		+	48	0.18
Y14-1550	+		40	1.22
Y14-1150	+		40	0.12
Y15-1050		+	45	0.01
Y15-550	+		45	0.06
Y17-1200		+	45	0.01
Z03-1500	+		48	0.18
Z06-700	+		40	0.35
Z09-1400		+	40	0.01
Z15-1050	+		45	3.17
Z16-2000		+	40	0.12
Z16-700		+	40	0.18
Z17-900	+		36	0.35
AA07-1700	+		45	0.06
AB04-900		+	40	1.23
AB07-800		+	45	0.18
AB12-450	+		40	0.12
AB16-1360	+		45	6.91**
AB16-980	+		45	3.81
AB18-610	+		40	0.58
AC14-680	+		45	0.18
AC15-1500	+		40	0.58
AC15-1200	+		40	2.90
AD02-950	+		45	1.22
AD04-1000		+	45	1.86
AD06-1500	+		45	0.36
AD12-800		+	45	0.03
AD15-800		+	45	0.06
AE02-1600	+		45	0.12
AE02-600		+	45	1.86
AE04-1650	+		40	0.18
AE04-800		+	40	2.60
AE07-1300	+		45	0.74
AE11-800		+	45	0.01
AE13-1020	+		45	0.26
AE16-2400		+	40	2.60
AE16-400	+		40	32.3**
AE17-2000	+		40	2.38
AE17-1000		+	40	0.01
AE17-800	+		40	0.03
AE17-650		+	40	0.26
AE20-100	+		40	0.35

*,** Significant at the 5% and 1% level, respectively

[a] Dominant allele present in the parent 'Térèse' (TE) or 'Torsdag' (TO) (isogenic to K586)

[b] Temperature of annealing (°C) used in the PCR reaction

[c] Chi-square value of goodness-of-fit to 1:1 ratio

Dirlewanger et al. (1994) and seven in Ellis et al. (1992)]. The RAPD marker NW04 and eight of the nine RFLPs fit the 1:1 expected ratio (Table 1). The position of the RFLP cDNA 136, which showed segregated distortion, has just been located. The different linkage groups were named according to the last version of the classical

genetic pea map (Weeden et al. 1996), and the linkage groups containing the classical markers *af* and *a* were numbered I and II, respectively.

With a linkage criteria LOD ≥ 5 and a distance of less than 30 cM, 237 loci were classified by two-point analysis into ten groups while 2 loci remained unlinked (T3-650, cDNA 187). When the minimum LOD score was lowered to 4, with a maximum map distance of 30 cM, nine linkage groups were obtained with two groups consisting of only 6 markers. By lowering the LOD score to 4, we could link linkage group containing the gene *le* to linkage group III (Fig. 1). Furthermore, 1 previously unlinked marker, cDNA 187, was included in linkage group V (Fig. 1). If we lowered the LOD score to 3 or below, the two small groups remained unlinked. Consequently, we preferred for the three-point analyses the nine groups obtained with linkage criteria LOD ≥ 4.0 and a distance ≤ 30 cM.

Construction of the map of population ('Térèse' × K586) RILs

The size of the nine linkage groups ranged from 20.1 cM to 187.1 cM, with a total genome coverage of 1104 cM. In the accompanying paper (Rameau et al. 1998), the previously unlinked marker, T3_650, was shown to map at the extremity of linkage group II on the other side of the branching gene *rms3* and at 9.5 cM from it. In the present RILs population, the two-point distance between T3_650 and O19_1050 was 35 cM, whereas it was at $9.5 + 21.5 = 31$ cM from O19_1050 in the F_2 population T2-30 × 'Torsdag'. If we include the RAPD T3_650 in group II, this group has a length of 164.2 cM, and the total length covered by the nine linkage groups is 1139 cM.

In linkage group VII, 2 RAPD loci that were amplified by the same primer (L13) mapped to the same position. These 2 bands (L13–1350 in K586, L13-1180 in 'Térèse') are either 2 alleles of a codominant RAPD locus or represent a case of strong competition between bands. RAPD polymorphisms are usually noted by the presence or absence of an amplification product from a single locus, which means that the RAPD technique tends to provide only dominant markers, although it is possible to obtain codominant RAPD markers (Kawchuk et al. 1994). The analysis of the primer L13 in an F_2 population obtained from the cross used to map a branching mutation (Rameau et al. 1998) provided some evidence in support of a codominant marker. This marker is noted L13_1350 in Fig. 1.

Screening of RAPD markers as common markers with the map of population (JI281 × JI399) RILs

Sixty-two primers which amplified polymorphic RAPD bands between 'Térèse' and 'Torsdag' and which were distributed over the different nine linkage groups were tested on JI281 and JI399. Amplified products generated from the four parental genotypes, 'Térèse', 'Torsdag', JI281 and JI399, were loaded next to each other for direct comparison of marker mobilities. Approximately half of them were monomorphic between JI281 and JI399, the band being absent in both genotypes in most cases; 25 primers gave the same (putatively) polymorphic band between JI281 and JI399. The amplified products obtained in JI281 and JI399 were assumed to be identical alleles at the same locus if they were generated with the same primer, if they showed the same electrophoretic mobility and if they mapped at the same genomic region. Of the 25 RAPD markers analyzed in the population JI281 × JI399 23 fitted these conditions (Table 3). Only 2 bands (G09_650 and AB7_800) were found to map at non-corresponding linkage groups, and a close re-examination of the amplified products loaded on a polyacrylamide gel confirmed that these 2 bands were not the bands segregating in the cross 'Térèse' × K586.

Alignment with the map of population (JI281 × JI399) RILs

In total, 32 common markers were used to align the two maps: the morphological marker *le*, 23 RAPD, seven RFLPs and the PCR marker UNI. The order of markers is quite well conserved between the two maps while the lengths of the different linkage groups are higher for the map of population JI281 × JI399, in particular for linkage group III (Fig. 1).

Three RAPD markers (K2_800, P11_600 and W19_1900) which mapped to one of the two small linkage groups had a distorted segregation ratio in the population JI281 × JI399 (Table 3), but they were approximately located at the top of linkage group I of this cross. Two markers (Vc5 and E16_650) located on the other small linkage group of 6 markers mapped to the top of linkage group III of the map of population JI281 × JI399 (Ellis et al. 1992).

Discussion

A pea genetic map covering 1139 cM and comprising 240 markers distributed over nine linkage groups has been developed using mainly RAPD markers. These markers have been selected for their repeatability between experiments, and we were able to align this map with the map of population JI281 × JI399 (Ellis et al. 1992; Hall et al. 1997a; Ellis et al. 1998) by using essentially RAPD markers. These RAPD markers could be used from one cross to another provided the amplified products generated from the two parents of a cross were loaded on a gel next to the

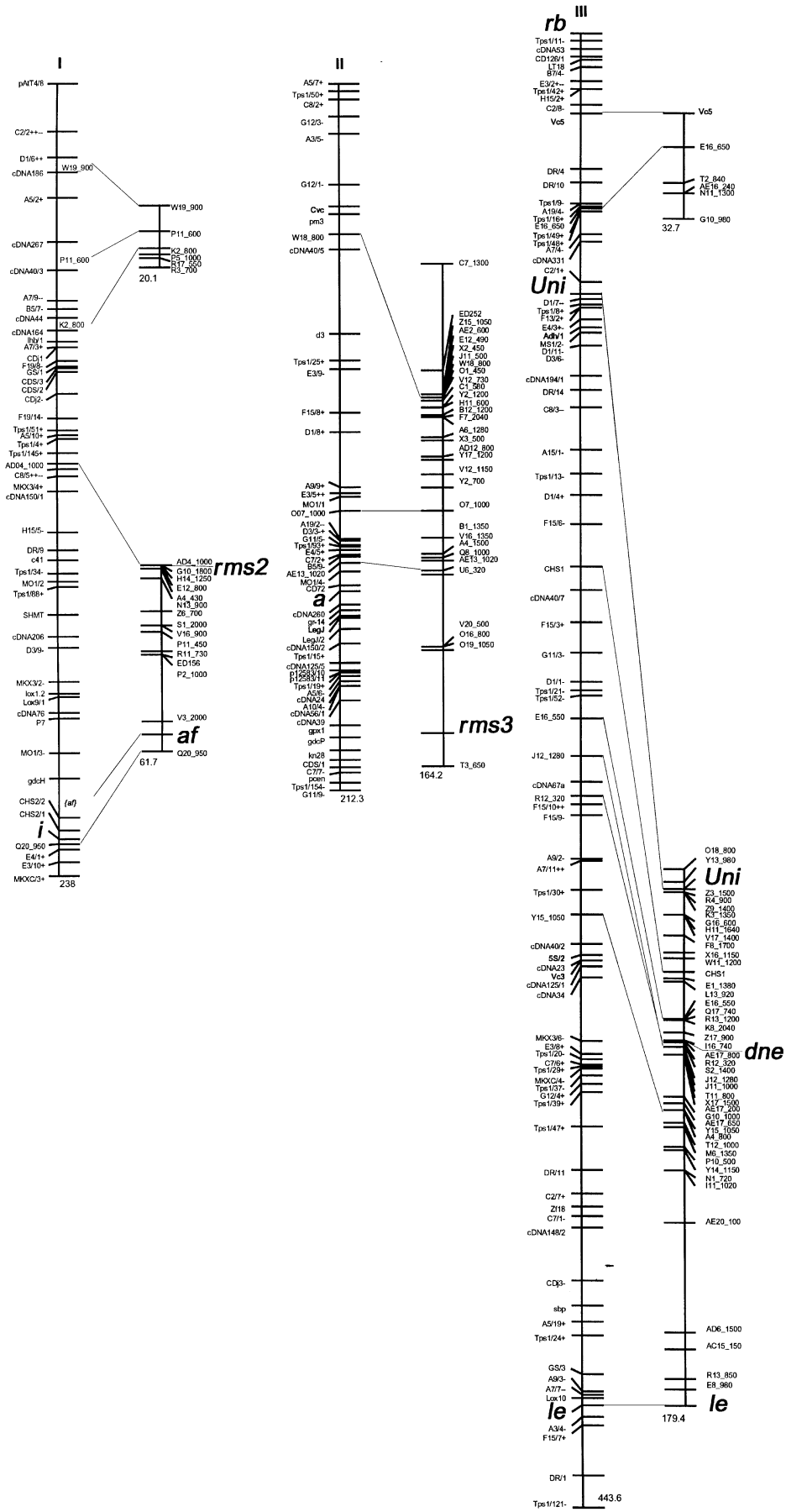

Fig. 1 Alignment of the two linkage maps presented for each of the classical linkage groups as derived from the population (JI281 × JI399) RILs (on the *left*) and from the population ('Térèse' × K586) RILs (on the *right*). For the map of population (JI281 × JI399) RILs, there are 357 markers covering 1881 cM plus 6 common markers assigned an approximate position because of a segregated distortion (LOD score < 3). For the map of population ('Térèse' × K586) RILs, there are 240 markers covering 1139 cM in nine linkage groups. Common markers are connected by *thin lines* between the two maps. The map length in Haldane units is given at the *bottom* of each linkage group. Classical markers (Ellis et al. 1992; Rameau et al. 1998) are written in *large*, *boldface letters*

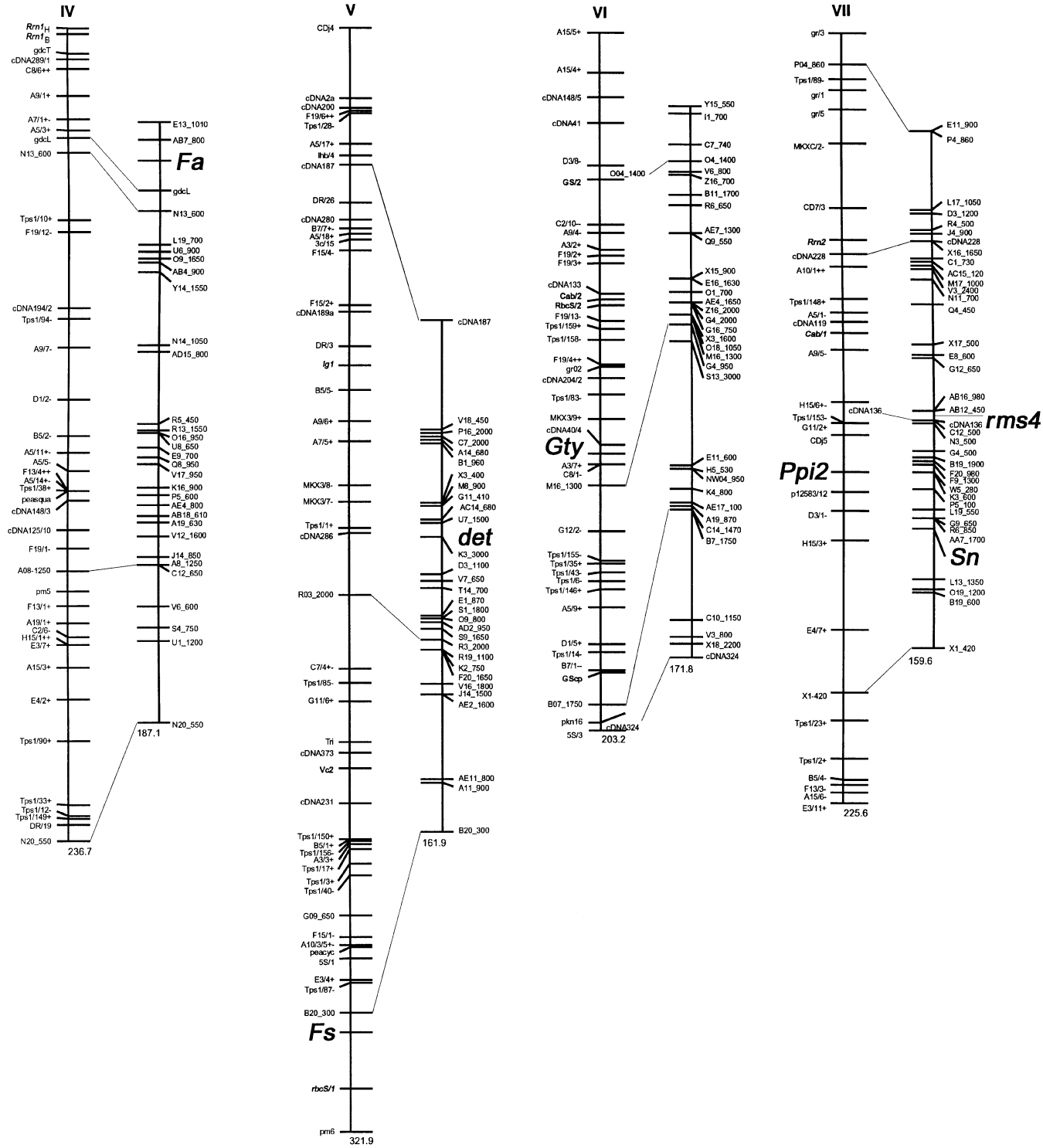

Fig. 1 Continued (See page 911 for legend)

amplified products generated from 'Térèse' and 'Torsdag'. These two genotypes are available on request. Recently, another pea linkage map has been generated with PCR-based markers (Gilpin et al. 1997), and it also includes several RAPD markers. To know if the RAPD marker O7_990 located on linkage group IA/II of their map corresponds to the RAPD marker O7_1000 on linkage group II of our map, the amplification products of the DNA of the four parents with the primer O7 should be compared.

Table 3 List of RAPD markers used as common markers

Linkage group	Marker	TE[a]	TO[a]	JI281[a]	J1399[a]	Chi^{2}[b]
I	K2-800		+	+		8.2
I	P11-600		+		+	11.8
I	Q20-950		+		+	0.1
I	W19-900	+			+	11.9
I	AD04-1000		+	+		1.8
II	O07-1000	+		+		0.2
II	W18-800	+		+		1.5
II	AE13-1020	+			+	0.8
III	E16-650		+		+	0.2
III	E16-550		+		+	1.8
III	J12-1280		+		+	0.4
III	R12-320	+			+	0.5
III	Y15-1050		+		+	0.5
IV	A08-1250	+			+	0.7
IV	N13-600	+			+	0.5
IV	N20-550		+	+		0.0
V	R03-2000		+		+	0.6
V	B20-300		+	+		0.2
VI	B07-1750	+			+	0.0
VI	M16-1300	+			+	0.0
VI	O04-1400	+		+		2.7
VII	P04-860		+		+	0.5
VII	X01-420	+		+		1.2

[a] Dominant allele present in the parent 'Térèse' (TE) or 'Torsdag' (TO), JI281 or JI399

[b] Chi-square value of goodness-of-fit to 1:1 ratio

A number of problems may arise with the integration of data from several crosses, such as: (1) correspondance between molecular markers scored in different crosses (see above), (2) variation among different crosses in the intensity of linkage between markers or (3) variation in linkage associations between markers. The latter is particularly evident for the garden pea where translocation events are relatively frequent and are presumably the cause of a number of contradictory results concerning linkage relationships between markers (Folkeson 1990). There have been considerable changes in the pea genetic map since the first publications (Lamprecht 1948, 1961; Blixt 1972, 1977). With the advent of new molecular techniques, isozyme and RFLP markers have been added (Weeden and Marx 1987; Weeden and Wolko 1990), and two RFLP maps have been generated (Ellis et al. 1992; Dirlewanger et al. 1994). In 1993, a revised linkage map of pea was published (Weeden et al. 1993) which described the difficulties in combining all the known markers identified in pea into a general linkage map. In particular, several of the classical linkage groups were split because of uncertainties in their integrity (Kosterin and Rozov 1993). Indeed, Paruvangada et al. (1995) suggested that the classical linkage groups IA and II may be on the same chromosome. This change has been confirmed by Hall et al. (Hall et al. 1997a, b) and adopted in the last version of the classical pea linkage map (Weeden et al. 1996).

The present work confirmed an important breakthrough in the pea map recently mentioned in Gilpin et al. (1997) and Ellis et al. (1998); that is, the association of the classical linkage segment IVB carrying *le*, the gene controlling internode length (Mendel 1866; Ingram et al. 1983), with linkage group III. In the map of population JI281 × JI399, the addition of PCR markers corresponding to insertions of the *Ty1-copia*-like class of retrotransposons allowed the joining of these two groups (Ellis et al. 1998). In our cross, the use of a LOD ≥ 4 generated nine linkage groups with the group carrying *le* joining the group III carrying *Uni* and *Dne* (Rameau et al. 1998).

The results presented here and in the accompanying paper are in good accordance with the pea genetic maps presented in Weeden et al. (1993, 1996). In Rameau et al. (1998), detailed alignments of the different maps obtained from the population of RILs in the present study and from different F_2 populations are shown. With these alignments, we were able to assign six of our nine linkage groups to the classical map: the genes *Rms2* and *Af* on linkage group I, the genes *Rms3* and *A* on linkage group II, *Dne* on linkage group III, *Fa* on linkage group IV, *Det* on linkage group V and *Rms4* and *Sn* on linkage group VII. The alignment of the map of population ('Térèse' × K586) RILs with the map of population (JI281 × JI399) RILs confirmed these associations and assigned the two small groups of 6 markers to linkage groups I and III.

Group I carries the RFLP marker ED156 which has been mapped in Dirlewanger et al. (1994) on the same linkage group as *fw*, a gene conferring resistance to *Fusarium oxysporum* f. sp. *pisi* race 1. This gene was assigned to chromosome 4 in Wells et al. (1949). Other markers found linked to *fw* in Dirlewanger et al. (1994) should be mapped to confirm or discount this association of *fw* to linkage group I. The other RFLP, ED252, located in Dirlewanger et al. (1994) at 16 cM from *mo*, mapped to our linkage group II which has been shown to carry the branching gene *Rms3* (Rameau et al. 1998). This result is in accordance with the linkage associations between *mo*, *k* and *wb* (Marx and Provvidenti 1979) and with the classical map (Weeden et al. 1993, 1996). The RAPD marker $NW04_{950}$, which has been mapped to linkage group VI by Timmerman et al. (1993) at approximately 23 cM from the gene *sbm-1* conferring resistance to the pea seed-borne mosaic virus, mapped also to our linkage group VI.

The linkage distance covered in the present map, 1139 cM, is substantially less than that of the map of population JI281 × JI399, which currently covers 1881 cM with 355 markers. This is still true if we add 200 cM for the two gaps of linkage groups I and III. RILs have been used with the same mapping function (Haldane) for both maps. Although there are several differences between the work described herein and that

of Ellis et al. (1992) (Hall et al.1997b; Ellis et al. 1998), it remains difficult to explain the cause(s) of the different map lengths obtained in the two studies. The higher number of RILs (139 compared with 71) does not explain the difference in map length as the higher number would tend to cause an increase rather than a decrease in map length. In the same way, the number of markers can not be an explanation as the map length was already higher in Ellis et al. (1992) with 151 RFLP markers covering 1700 cM. It is possible that differences in linkage intensity in different crosses can explain such a difference in map length. A study of the relatedness of the various pea lines used in Ellis et al. (1992), and based on 72 RFLP markers, shows that the line JI281 (from Ethiopia) is genetically distant in comparison with other lines (Ellis 1993). However, this factor would have tended to cause a shorter map in the wider cross. Indeed, among the different factors which tend to lead to map inflation, misclassification is one of the most serious causes (Lincoln and Lander 1992; Sybenga 1996). This has been discussed in Ellis et al. (1992) in relation to the consequences of DNA methylation at the *Adh* locus in linkage group III. This may explain in part the particular high difference in map lengths of this linkage group (444 cM vs. 33 + 180 cM).

On the basis of cytogenetic studies in which chiasma frequency per meiosis is estimated, a map length of about 700–800 cM is expected (Hall et al. 1997a, b). So the length of our RAPD map, which is closer even than the slightly shorter map of the cross JI15 × JI399 also used in Hall et al. (1997b), is more consistent with this estimate but is still higher than expected. This discrepancy between map lengths and chiasma counts has been discussed in Sybenga (1996) and Hall et al. (1997b) and is still under investigation.

The ease with which the RAPD markers can be transferred between the 'Térèse' × K586 and JI281 × JI399 populations is notable and commends this marker type to pea genetics. The integration of these maps increases the effective marker density for the pea linkage map; it is particularly interesting for linkage group II where the high density of markers in the top (bottom) part of one map compensates the low density in the other map (Fig. 1). This RAPD-based map will be used in several breeding programs of INRA where different populations of RILs are being developed to look for QTLs involved in variation of characters such as resistance to anthracnose and cold resistance. When possible, crosses involve the cv 'Térèse' to facilitate the use of the RAPD markers mapped in this study.

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References

Arumingtyas EL, Floyd RS, Gregory MJ, Murfet IC (1992) Branching in *Pisum*: inheritance and allelism tests with 17 *ramosus* mutants. Pisum Genet 24: 17–31

Blixt S (1972) Mutation genetics in *Pisum*. Agric Hortic Genet 30: 1–293

Blixt S (1977) The gene symbols of *Pisum*. Pisum Newsl 9 [Suppl]: 1–59

Church G, Gilbert W (1984) Genomic sequencing. Proc Natl Acad Sci USA 81: 1991–1995

Dirlewanger E, Isaac PG, Ranade S, Belajouza M, Cousin R, de Vienne D (1994) Restriction fragment length polymorphism analysis of loci associated with disease resistance genes and developmental traits in *Pisum sativum* L. Theor Appl Genet 88: 17–27

Doyle JJ, Doyle JL (1987) Isolation of plant DNA from fresh tissue. Focus 12: 13–15

Ellis THN (1993) The nuclear genome. In: Casey R, Davies DR (eds) Peas: genetics, molecular biology and biotechnology. CAB Int, Wallingford, UK, pp 13–47

Ellis THN, Turner L, Hellens RP, Lee D, Harker CL, Enard C, Domoney C, Davies DR (1992) Linkage maps in pea. Genetics 130: 649–663

Ellis THN, Poyser SJ, Knox MR, Vershinin AV, Ambrose MJ (1998) Ty1-copia class retrotransposon insertion site polymorphism for linkage and diversity analysis in pea. Mol Gen Genet (in press)

Ellsworth DL, Rittenhouse KD, Honeycutt RL (1993) Artifactual variation in randomly amplified polymorphic DNA banding patterns. Biotechniques 14: 214–217

Folkeson D (1990) A revised genetic map of *Pisum sativum*. PhD thesis, University of Lund Sweden

Gilpin BJ, McCallum JA, Frew TJ, Timmerman-Vaughan GM (1997) A linkage map of the pea (*Pisum sativum* L.) genome containing cloned sequences of known function and expressed sequence tags (ESTs). Theor Appl Genet 95: 1289–1299

Hall KJ, Parker JS, Ellis THN (1997a) The relationship between genetic and cytogenetic maps of pea. I. Standard and translocation karyotypes. Genome 40: 744–754

Hall KJ, Parker JS, Ellis THN, Turner L, Knox MR, Hofer JMI, Lu J, Ferranndiz C, Hunter PJ, Taylor JD, Baird K (1997b) The relationship between genetic and cytogenetic maps of pea. II. Physical maps of linkage mapping populations. Genome 40: 755–769

Hengen PN (1995) Cloning PCR products using T-vectors. TIBS 20: 85–86

Hofer J, Turner L, Hellens R, Ambrose M, Matthews P, Michael A, Ellis N (1997) *UNIFOLIATA* regulates leaf and flower morphogenesis in pea. Curr Biol 7: 581–587

Ingram TJ, Reid JB, Potts WC, Murfet IC (1983) Internode length in *Pisum*. IV. The effect of the *Le* gene on gibberellin metabolism. Physiol Plant 59: 607–616

Jones CJ, Edwards KJ, Castaglione MO, Winfield MO, Sala F, van de Wiel, Bredemeijer G, Vosman B, Matthes M, Daly A, Brettschneider R, Bettini P, Buiatti M, Maestri E, Malcevschi A, Marmiroli N, Aert R, Volckaert G, Rueda J, Linacero R, Vazquez A, Karp A (1997) Reproducibility testing of RAPD, AFLP and SSR markers in plants by a network of European laboratories. Mol Breed 3: 381–390

Kawchuk LM, Lynch DR, Hachey J, Bains PS, Kulcsar F (1994) Identification of a codominant amplified polymorphic DNA marker linked to the *verticillium* wilt resistance gene in tomato. Theor Appl Genet 89: 661–664

Kosterin OE, Rozov SM (1993) Mapping of the new mutation *blb* and the problem of integrity of linkage group I. Pisum Genet 25: 27–31

Lamprecht H (1948) The variation in linkage and the course of crossing over. Agric Hortic Genet 6: 10–48

Lamprecht H (1961) Die Genenkarte von *Pisum* bei normaler Struktur der Chromosomen. Agric Hortic Genet 19 : 360–401

Lander ES, Green P, Abrahamson J, Barlow A, Daly M, Lincoln S, Newberg L (1987) MAPMAKER: an interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. Genomics 1 : 174–181

Lincoln SE, Lander ES (1992) Systematic detection of errors in genetic linkage data. Genomics 14 : 604–610

Lincoln S, Daly M, Lander ES (1992) Constructing genetic maps with MAPMAKER/EXP 3.0. Whitehead Institute Technical Report, 3rd edn. Whitehouse Technical Institute, Cambridge, Mass.

Marx GA, Provvidenti R (1979) Linkage relation of *mo*. Pisum Newsle 11 : 28–29

Mendel G (1866) Versuche über Pflanzen-hybriden. Verh Naturf Verein Brünn 4 : 3–47

Michelmore RW, Paran I, Kesseli RV (1991) Identification of markers linked to disease resistance genes by bulked segregant analysis : a rapid method to detect markers in specific genomic regions by using segregating populations. Proc Natl Acad Sci USA 88 : 9828–9832

Murfet IC, Reid JB: (1993) Developmental mutants. In : Casey R, Davies DR (eds) Peas: genetics, molecular biology and biotechnology. CAB Int, Wallingford, UK, pp 165–216

Paruvangada VG, Weeden NF, Cargnoni T, Yu J, Gorel′ F, Frew T, Timmerman-Vaughan GM, Mc Callum J (1995) *Enod2* exhibits linkage with *a* (linkage group IA) and *Fum* (linkage group II). Pisum Genet 27 : 12–13

Powell W, Morgante M, Andre C, Hanafey M, Vogel J, Tingey S, Rafalski A (1996) The comparison of RFLP, RAPD, AFLP and SSR (microsatellite) markers for germplasm analysis. Mol Breed 2 : 225–238

Rameau C, Bodelin C, Cadier D, Grandjean O, Miard F, Murfet IC (1997) New *ramosus* mutants at loci *Rms1*, *Rms3* and *Rms4* resulting from the mutation breeding program at Versailles. Pisum Genet 29 : 7–12

Rameau C, Dénoue D, Fraval F, Haurogné K, Josserand J, Laucou V, Batge S, Murfet IC (1998) Genetic mapping in pea. 2. Identification of RAPD and SCAR markers linked to genes affecting plant architecture. Theor Appl Genet 97 : 916–928

Reid JB, Ross JJ (1993) A mutant-based approach, using *Pisum sativum*, to understand plant growth. Int J Plant Sci 154 : 22–34

Sybenga J (1996) Recombination and chiasmata : few but intriguing discrepancies. Genome 39 : 473–484

Thomas CM, Vos P, Zabeau M, Jones DA, Norcott KA, Chadwick BP, Jones JDG (1995) Identification of amplified restriction fragment polymorphism (AFLP) markers tightly linked to the tomato *Cf-9* gene for resistance to *Cladosporium fulvum*. Plant J 8 : 785–794

Timmerman GM, Frew TJ, Miller AL, Weeden NF, Jermyn WA (1993) Linkage mapping of *sbm*-1, a gene conferring resistance to pea seed- borne mosaic virus, using molecular markers in *Pisum sativum*. Theor Appl Genet 85 : 609–615

Timmerman GM, Frew TJ, Weeden NF, Miller AL, Goulden DS (1994) Linkage analysis of *er-1*, a recessive *Pisum sativum* gene for resistance to powdery mildew fungus (*Erysiphe pisi* D.C.). Theor Appl Genet 88 : 1050–1055

Vos P, Hogers R, Bleeker M, Reijans M, van de Lee T, Hornes M, Fritjers A, Pot J, Peleman J, Kuiper M, Zabeau M (1995) AFLP: a new tool for DNA fingerprinting. Nucleic Acids Res 23 : 4407–4414

Weeden NF, Marx GA (1987) Further genetic analysis and linkage relationships of isoenzyme loci in the pea. J Hered 78 : 153–159

Weeden NF, Wolko B (1990) Linkage map for the garden pea (*Pisum sativum*) In: O'Brien SJ (ed) Genetic maps. Locus maps of complex genomes, 5th edn. Cold Spring Harbor Laboratory Press, New York, pp 6.106–6.112

Weeden NF, Swiecicki WK, Ambrose M, Timmerman GM (1993) Linkage groups of pea. Pisum Genet 25 : 4 (and cover)

Weeden NF, Swiecicki WK, Timmerman-Vaughan GM, Ellis THN, Ambrose M (1996) The current pea linkage map. Pisum Genet 28 : 1–4

Wells DG, Hare W, Walker W (1949) Evaluation of resistance and susceptibility in garden pea to near wilt in glasshouse. Phytopathology 39 : 771–779

Welsh J, McClelland M (1990) Fingerprinting genomes using PCR with arbitrary primers. Nucleic Acids Res 18 : 7213–7218

Williams JGK, Kubelik AR, Livak KJ, Rafalski JA, Tingey SV (1990) DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res 18 : 6531–6535