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# Genetic mapping in pea. 2. Identification of RAPD and SCAR markers linked to genes affecting plant architecture

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**Abstract** Random amplified polymorphic DNA (RAPD) markers linked to two morphological markers (*fa* and *det*), three *ramosus* genes (*rms2*, *rms3* and *rms4*) and two genes conferring flowering response to photoperiod in pea (*sn*, *dne*) were selected by bulk segregant analysis on $F_2$ populations. Two RAPD fragments were cloned and sequenced to generate the two SCAR markers V20 and S2 which are linked to *rms3* and *dne*, respectively. All these genes, except *rms2*, were previously located on the pea classical linkage map. *Rms2* mapped to linkage group IB which contains the *afila* gene. Precise genetic maps of the regions containing the genes were obtained and compared to the RAPD map generated from the recombinant inbred-lines population of the cross Térèse × K586. This cross was chosen because several mutants were obtained from cultivars Térèse and Torsdag (K586 was derived from Torsdag). This collection of isogenic lines was used for the construction of $F_2$ mapping populations in which polymorphic RAPD markers were already known and mapped. Moreover, the well-known problem in pea of variability in the linkage associations between crosses was avoided. This work contributes to the precise integration between the classical map and the molecular maps existing in pea.

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## Introduction

In the pea (*Pisum sativum* L.), as in many other crops, there are important agronomic advantages to controlling plant architecture. The major problems of the pea crop are its relatively low and variable yields together with harvesting difficulties (Heath and Hebblewaite 1985). Agronomic studies in pea have shown that the number of stems bearing pods is an important yield component (Jeuffroy 1991; Doré 1992). The ideotype is not precisely defined between a plant with one main stem bearing several reproductive nodes, but with high competition between pods during seed filling, and a plant with several basal branches (secondary stems) each producing less reproductive nodes.

Selection towards a better standing ability and for yield components that minimize intra-plant competition between developing pods can be obtained by improved control of the branching habit and of the resource allocation between reproductive and vegetative growth. Several genes have already been identified in pea that control one or both of these features. Murfet and Reid (1993) described two categories of genes which influence branching in pea: genes identified because of their direct effect on apical dominance (*ramosus* genes) and genes identified for their effect on some other trait (e.g. some internode-length and flowering genes) but which have pronounced pleiotropic effects on branching.

Five *Rms* (*ramosus*) genes have been identified in pea (Blixt 1976; Apisitwanish et al. 1992; Arumingtyas et al. 1992) and several alleles are known for each locus (Symons and Murfet 1997). The *rms* mutants show increased basal and aerial branching with a reduction in height of the main stem. Because of the increased

aerial branching, these mutants are not appropriate for crop use. Nevertheless, they constitute a genetic resource of significant value and their use in physiological studies is contributing to a better understanding of apical dominance and of the role of auxin and cytokinin in the control of lateral bud initiation and subsequent growth (Beveridge et al. 1994, 1996, 1997 a, b).

In pea, the genes *Sn* (Barber 1959; Murfet 1971), *Dne* (King and Murfet 1985) and *Ppd* (Arumingtyas and Murfet 1994) control the flowering response to photoperiod. These genes also have a significant effect on the branching pattern. Photoperiodic types have a marked tendency to produce basal laterals, particularly under short days, whereas day-neutral plants usually produce only a single stem (Doroshenko and Rasumov 1929; Floyd and Murfet 1986; Murfet and Reid 1993). Moreover, the primary role of the product of these genes may be to direct assimilate flow within the plant (Beveridge et al. 1992; Reid and Murfet 1984). Consequently, these genes are of agronomic interest as they control assimilate partitioning between vegetative and reproductive growth. Combining-pea cultivars with the wild-type genotype *Sn Dne Ppd hr* are quantitative long-day plants, while early market cultivars with the mutant *sn* allele are day neutral types with a rapid life cycle (Murfet 1990; Murfet and Reid 1993). The mutant allele *dne* is leaky (Murfet 1989) and thus offers the potential to fill an intermediate niche (Murfet 1990).

In an accompanying paper, we developed a RAPD genetic map in pea from a population of 139 recombinant inbred lines (RILs) derived from the cross Térèse × K586 (Laucou et al. 1998). K586 is an increased branching mutant obtained from the cultivar Torsdag (Uzhintseva and Sidorova 1979). Térèse (dwarf, *le*) and Torsdag (tall, *Le*) are two combining-cultivars with a wild-type branching habit that have been used in different mutagenesis programs. In particular, several mutant alleles of the *rms* loci have been obtained from these two cultivars (Rameau et al. 1997; Symons and Murfet 1997). In the present study, several $F_2$ populations, generally derived from crosses involving Térèse and/or Torsdag isogenic lines, have been used to identify RAPD markers closely linked to seven genes affecting plant architecture: the two classical morphological markers *fa* and *det*, three *ramosus* genes (*rms2*, *rms3* and *rms4*) and two genes affecting sensitivity to photoperiod (*sn* and *dne*).

All of these genes (except *rms2*) have already been mapped on one of the pea linkage groups relative to classical markers (Weeden et al. 1993, 1996). Marx (1986) reported linkage between the genes *Det* (indeterminate/limited growth), *R* (smooth/wrinkled seeds) and *Bt* (blunt/pointed pod apex) which are located at the top of linkage group V, *Det* being about 22 map units from *Bt* and 7 from *R*. Makasheva and Drozd (1987) reported linkage between *Det* and *Tl* (tendrils/tendrilless) with a distance of 11 map units. The gene *Fa* (normal/fasciated stem), previously mapped on chromosome 4 by Blixt (1974), is reported to be linked to *Rrn1* (ribosomal DNA) at 18 map units (Polans et al. 1986). *Rrn1* has been mapped on linkage group IVA close to the RFLP marker pSTL (Ellis et al. 1992; Hall et al. 1997). The two *ramosus* loci *Rms3* and *Rms4* are located on linkage groups II and VII, respectively. *Rms3* has been linked to *A* at about 25 cM and *Rms4* to *Aat-m* at 4 cM (Poole et al. 1993). A close linkage between *Dne* and *st* (small stipules) was reported by King and Murfet (1985). Later, *Dne* was more precisely located in group III between *st* and *b* (color of the flower) at 5 and 27 map units, respectively (Murfet 1987). The other flowering gene, *Sn*, has been mapped close to the amylase locus *Amy1* (Weeden et al. 1988) on linkage group VII. This has been confirmed recently by Murfet and Sheriff (1996) who reported its location between two other isozyme loci, *Aldo* and *Gal2*, at 13 and 25 units, respectively.

In the present paper, we report new and precise relationships between morphological, physiological and RAPD markers in pea. The objectives are to identify closely linked molecular markers to genes of potential agronomic interest that can be used easily in crosses and to integrate our RAPD map (Laucou et al. 1998) with the classical pea genetic map.

## Materials and methods

### Segregating populations

For a given gene, $F_2$ populations were used for mapping (Table 1). The lines JI2121 (*det*) and JI814 (*fa*) were provided by M. Ambrose (John Innes Center, Norwich, UK). Several lines come from the collection at Hobart, Australia, in particular several mutant lines derived from cv Torsdag (Uzhintseva and Sidorova 1979): namely, the day-neutral mutant K218 (*Lf E Sn dne Ppd hr*) (King and Murfet 1985), and branching mutants K524 (*rms2-1*) and K564 (*rms3-2*). The line WL6042 (*rms3-3*) was derived, according to the Nordic Gene Bank (Weibullsholm) list, from the dwarf (*le*) cv Meteor but more likely came from cv Raman (Arumingtyas et al. 1992). The line HL59 (*lf E sn Dne Ppd hr*) is a standard line for the early day neutral phenotypic class (Murfet 1985). The lines T2-30 (*rms3–4*) and M3T-946 (*rms4-3*) were obtained at Versailles following treatment of cv Térèse with ethyl methane sulfonate (Rameau et al. 1997). Térèse and Torsdag belong to the late-flowering phenotypic class with a quantitative response to photoperiod (*Lf E Sn Dne Ppd hr*). SGE80 is the type line for the gene *blb* (*bulbosus*) mapped on linkage group IA (Kosterin and Rozov 1993).

### Bulk segregant analysis

The strategy of bulk segregant analysis developed by Michelmore et al. (1991) was used to find RAPD markers linked to the gene of interest. All $F_2$ plants were genotyped by growing on $F_3$. $F_2$ individuals heterozygous for the mapped gene were excluded from the pools to allow the identification of RAPD markers both in coupling and in repulsion (i.e. RAPD bands from both parents). Seven to seventeen $F_2$ plants were selected for each bulk DNA sample (Table 1). The primers that revealed potential polymorphisms between the bulks were confirmed first on the $F_2$ individuals used in

**Table 1** List of the genes mapped and description of the corresponding $F_2$ populations used in the bulk segregant analysis

| Mutation | Phenotype | Parents used in the cross | $F_2$ progeny size | Number of plants/pool | |
|---|---|---|---|---|---|
| | | | | aa | AA |
| *det* | Determinate growth | JI2121<br>Térèse | 142 | 10 | 9 |
| *fa* | Fasciated stem | JI814<br>Térèse | 124 | 10 | 9 |
| *sn* | Photoperiod insensitivity | HL59<br>Térèse | 98 | | |
| *dne* | Photoperiod insensitivity | K218 (ex Torsdag)<br>Térèse | 108 | 15 | 17 |
| *rms2* | Increased branching | K524 (ex Torsdag)<br>Térèse | 199 | 12 | 12 |
| *rms3* | Increased branching | WL6042 (ex Raman)<br>Térèse | 57 | 9 | 7 |
| | | T2-30 (ex Térèse)<br>Torsdag | 182 | 11 | 11 |
| | | K564 (ex Torsdag)<br>SGE80 | 128 | | |
| *rms4* | Increased branching | M3T-946 (ex Térèse)<br>Torsdag | 197 | | |

the two pools and then by segregation analysis. The choice of primers to be tested between the two bulks varied in time according to the progression of our RAPD map and the cross used; e.g. if the two parents were isogenic to Térèse and Torsdag, only primers known to give polymorphic bands between those two parents were utilized.

### $F_2$ and $F_3$ screening for *Dne*

One hundred and eight $F_2$ plants from the cross Térèse × K218, the parent lines and $F_1$ plants were grown in individual pots in a growth chamber. Tests on $F_2$ plants were conducted in two separate experiments. All plants received 10 h of light per day (cool white light from bulbs providing 40–70 μmol $m^{-2}$ $s^{-1}$ at the pot top; MBF 400 W, Claude, Courbevoie, France) at a temperature between 20 and 24°C. After observation of the first open flower, the $F_2$ plants were transferred to glasshouse conditions under a 16-h photoperiod for the production of $F_3$ seeds. When available, 16 $F_3$ plants per $F_2$ plant were grown in the same conditions as for the $F_2$, four per 5-l pot. Only eight $F_3$ plants per $F_2$ mutant plant were grown to confirm the mutant phenotype and to act as controls for the $F_3$ screening. All data for flowering node and time were obtained from the main shoot. Node counts were commenced from the first scale leaf as node 1. Flowering time was taken as the number of days from sowing to first open flower.

### $F_2$ and $F_3$ screening for *Sn*

The $F_2$ plants were grown in a heated glasshouse under long days (16-h photoperiod) to give sufficient $F_3$ seeds. The natural day length was extended and supplemented during the day with sodium lamps (SON/T AGRO 400W, Philips, Ivry/Seine, France). Twenty $F_3$ seeds per family were analysed for several characters under short-day conditions in a heated glasshouse during winter when the natural day length did not exceed 10 h. Control lines of known flowering genotype were grown at the same time. The natural day light was supplemented by light from sodium lamps. Several variables were recorded for $F_2$ and $F_3$ plants: height of the plant, days from sowing to first open flower, node of flower initiation, node of first pod, number of reproductive nodes on the main stem, peduncle length and, for $F_3$ plants only, the flower/leaf relativity index as defined by Murfet (1982).

### $F_2$ and $F_3$ screening for the other genes

The five mutations *det*, *fa*, *rms2*, *rms3* and *rms4*, all showed clear expression in the segregating populations. Generally, 20 $F_3$ individuals were observed in the greenhouse to confirm the $F_2$ genotype.

### DNA extraction

Stipules and/or leaflets (in an *Af* background) were harvested from $F_2$ plants, immediately frozen in liquid nitrogen and kept at −80°C until DNA extraction. Genomic DNA was extracted using the CTAB procedure modified from Doyle and Doyle (1987), see Laucou et al. (1998).

### RAPD procedure

The PCR reactions were performed in a 25-μl vol of 10 mM Tris-HCl (pH 9), 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X100, 0.2 mg/ml BSA, 100 μM of each dNTP, 20 ng of 10-mer primer, 10 ng of genomic DNA and 0.5 units of *Taq* DNA polymerase (Appligene Oncor, France).

Amplification was performed in a Perkin-Elmer Cetus DNA Thermal Cycler 480 or a PTC 100 Thermocycler (MJ Research, Bioconcept, Switzerland). The decamer primers for RAPD markers were obtained from Operon Technologies (Alameda, Calif.; Operon kits A to AE). PCR conditions were: 94°C for 5 min followed by 45 cycles consisting of 1 min at 94°C, 1 min at 40°C or 45°C, and 2 min at 72°C. Amplified products were separated by electrophoresis at 4 V/cm in a 1.4% agarose gel stained with ethidium bromide and visualised under UV light.

## Cloning and sequencing of RAPD products

The amplified products of the linked RAPD bands were excised from the agarose gel and the DNA was purified by electroelution into dialysis bags using the protocol described by Sambrook et al. (1989). The purified DNA was ligated into a pBluescript II SK (−) vector (Stratagene, La Jolla, Calif.) that had been restricted with *Eco*RV using the T-A overhang cloning procedure described by Marchuk et al. (1991). The vectors were introduced into *Escherichia coli* DH5α by electroporation. White clones were verified by hybridization to Southern blots of $F_2$ individuals that segregated for the progenitor RAPD markers. Both ends of each DNA insert were sequenced on an ABI 373 automated sequencer (Applied Biosystems, Inc) using a *Taq* DyeDeoxy™ Terminator Cycle Sequencing kit (Applied Biosystems) as recommended by the manufacturer. Two specific primers of 24 bases in length were designed and synthesised by Oligoexpress (Montreuil, France). The primers sequences are, for S2_1900: upper primer (5′ TATTGCTATTTTGTAGGTTTGCGT 3′), lower primer (5′ ACCAGACTACTGTTGTTTGTCACA 3′); and for V20_1100: upper primer (5′ ACAAATAAATCAAACGCA-ACGAAT 3′), lower primer (5′ CCAACCATACTCCCTCT AAC-CATC 3′).

## Amplification of products using the specific primer pair

Amplification of the specific markers was done in a 25-µl vol containing 10 mM Tris-HCl (pH 9), 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X100, 0.2 mg/ml BSA, 100 µM of each dNTP, 20 pmol of 24-mer primer, 10 ng of genomic DNA and 0.5 units of *Taq* DNA polymerase (Appligene Oncor, France). Amplifications were performed in a Perkin-Elmer Cetus DNA Thermal Cycler 480 for 35 cycles after denaturation for 5 min at 94°C. Each cycle consisted of 1 min at 94°C, 1 min at 52 or 55°C, and 1 min at 72°C. Amplification products were resolved in a 1.4% agarose gel. The selected 24-mer primers were tested on the parents. The SCAR products amplified from both parents were digested with different restriction endonucleases which recognize 4- and 5-bp sequences in order to uncover polymorphisms in fragment length.

## Linkage analysis

The problem of mapping dominant markers, and in particular RAPD markers, using repulsion $F_2$ matings has been discussed by many authors (Tingey et al. 1992; Säll and Nilsson 1994;

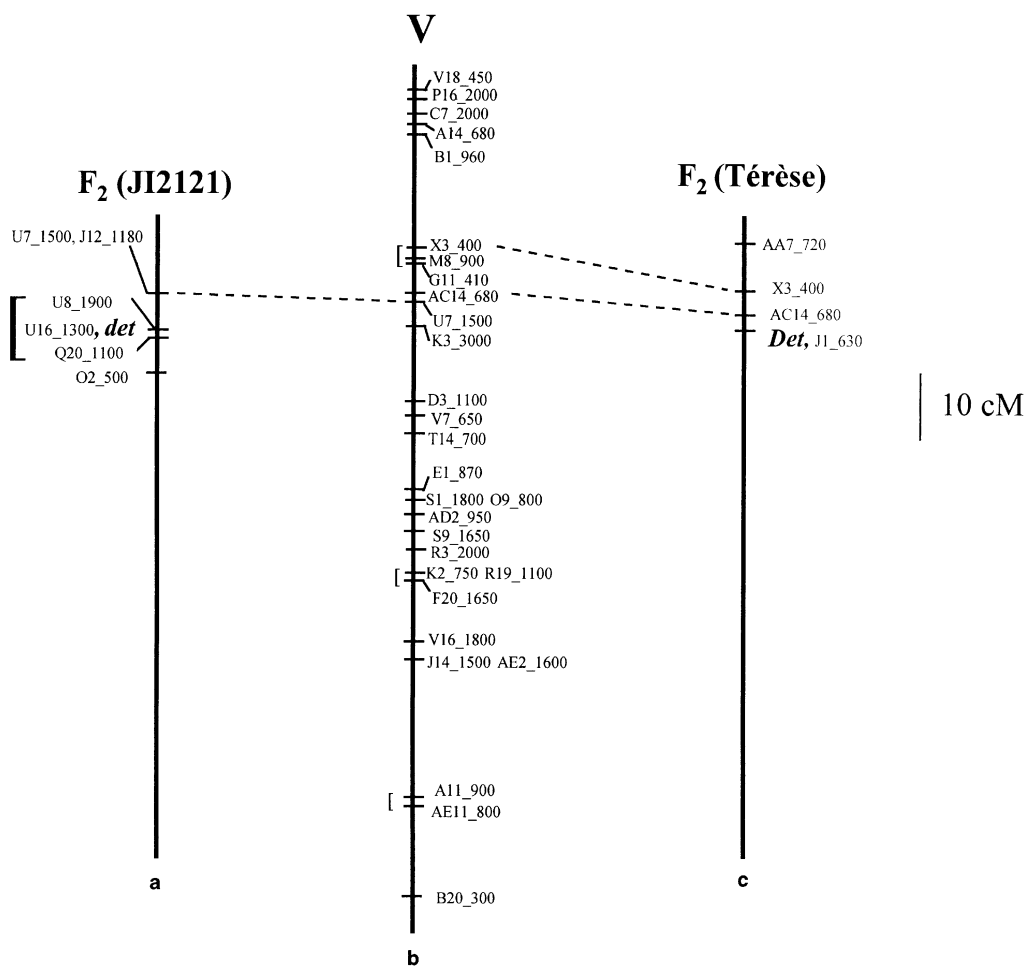


**Fig. 1 a–c** Linkage maps of the region surrounding the gene *Det* derived from analysis of the $F_2$ population Térèse × JI2121. **a** Markers specific to JI2121. **c** Markers specific to Térèse. **b** Linkage map of linkage group V derived from analysis of the RILs population, from Laucou et al. (1998). Groups of markers with a *square bracket* to the left cannot be ordered.

Knapp et al. 1995). Nevertheless, valid maps of dominant markers can be built by using two sub-sets of markers linked in coupling. Consequently, for a given $F_2$ population, it was more appropriate to construct two separate 'coupling' maps, one map with markers specific to parent 1 and the other specific to parent 2. The gene of interest, which was analysed as a co-dominant marker, has been considered in the two sub-sets of RAPD dominant markers.

The putatively linked markers were screened in the $F_2$ population to confirm their linkage to the gene. Chi-square tests were used to test for departure from the expected 3 : 1 or 1 : 2 : 1 ratio. Segregation data were analysed using MAPMAKER (Lander et al. 1987), see Laucou et al. (1998).

### Selection of RAPD polymorphisms for bulk segregant analysis

Initially 620 primers (Kits A–AE) were screened on Térèse and Torsdag for the construction of the RAPD map. Of the 620 primers screened, 240 were not used in the bulk segregant analysis study because they gave no, or only weak, amplification or a smear profile. Of the 380 remaining primers, 160 showed at least one clear and reproducible polymorphic band between Térèse and Torsdag and the RAPD markers were mapped in linkage groups (Laucou et al. 1998).

For four of the seven classical genes mapped in this study, we have used an $F_2$ population derived from the cross between the corresponding mutant line in the Torsdag (or Térèse) genetic background and Térèse (or Torsdag). In this case, only the 160 primers known to give polymorphic RAPD markers between Térèse and Torsdag were used; less primers were employed for genes mapped when the RAPD map was achieved (*rms2*, *rms4*): bulk segregant analysis was carried out with primers giving RAPD markers spread along the different linkage groups. For the other three genes where one parent of the $F_2$ population used was not isogenic to Térèse or Torsdag (*det*, *fa*, *sn*), the 380 primers were tested.

## Results and discussion

### *Det* locus (Fig. 1)

Segregation for *Det* fitted the expected 1 : 2 : 1 ratio (32 : 77 : 33, $\chi^2 = 1.04$). Screening of 380 primers on bulked samples of *det det* vs *Det Det* homozygotes revealed ten reproducible polymorphisms: six in repulsion with *Det*, four in coupling. Segregation analysis confirmed the linkage of these ten potential polymorphisms with the gene *Det*. Among these ten RAPD markers, only three were polymorphic between Térèse and Torsdag (U7_1500, AC14_680, X3_400) and were mapped on the RILs. No other markers located on linkage group V, and not too far from *Det*, were found to be polymorphic between Térèse and JI2121 (AE2_1600 is specific to Térèse but this marker can not be linked to J1_630 which should be the nearest marker in coupling phase). Closely linked markers at less than 2 cM from *det* have been found (U16_1300, Q20_1100, U8_1900) (Fig. 2 a).

### *Fa* locus (Fig. 3)

The $F_2$ population of the cross Térèse × JI814 segregated in agreement with a 1 : 2 : 1 ratio for *Fa* (38 : 57 : 29, $\chi^2 = 2.11$). Ten homozygous *fa* plants and nine homozygous *Fa* plants were used for pools. Six RAPD markers were found to be polymorphic between the two pools, five in coupling and only one in repulsion. These markers were confirmed to be linked to *Fa*; the marker in repulsion, Q11_1380 (Fig. 2 b), at 8.6 cM from *fa*. Four RAPDs were also mapped on the RILs and allow the alignment of the $F_2$ map with the RILs map (Fig. 3).

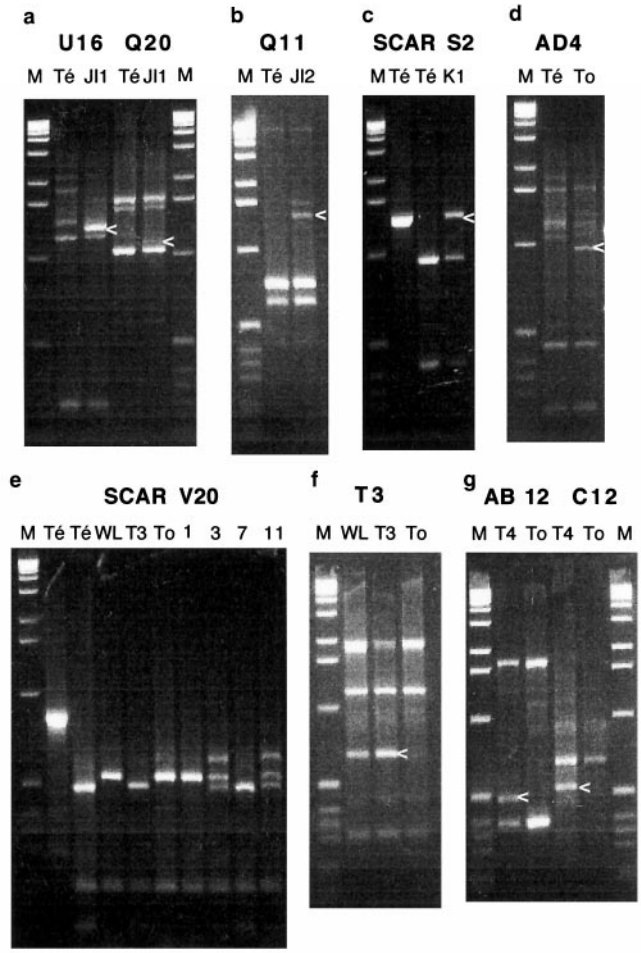


**Fig. 2 a–g** Agarose gels showing the result of PCR amplification of DNA samples from parents of $F_2$ populations. **a** Polymorphisms linked to the gene *det* between Térèse (*Té*) and JI2121 (*JI1*). **b** Polymorphism linked to the gene *fa* between Térèse (*Té*) and JI814 (*JI2*). **c** Polymorphism linked to the gene *dne* between Térèse (*Té*) (*lane 2*: no digestion, *lanes 3 and 4*: digestion with *Cfo*I) and K218 (*K1*). **d** Polymorphism linked to *rms2* between Térèse (*Té*) and Torsdag (*To*). **e** and **f** Polymorphisms linked to *rms3* between WL6042 (*WL*), T2-30 (*T3*) and Torsdag (*To*). Segregation of SCAR V20; *lane 2*: no digestion, *lanes 3–10*: digestion with *Hin*fI. *Lanes 7–10*: four $F_2$ individuals. **g** Polymorphisms linked to *rms4* between M3T-946 (*T4*) and Torsdag (*To*) *M* = 1-kb ladder. *Arrows* point to polymorphic fragments

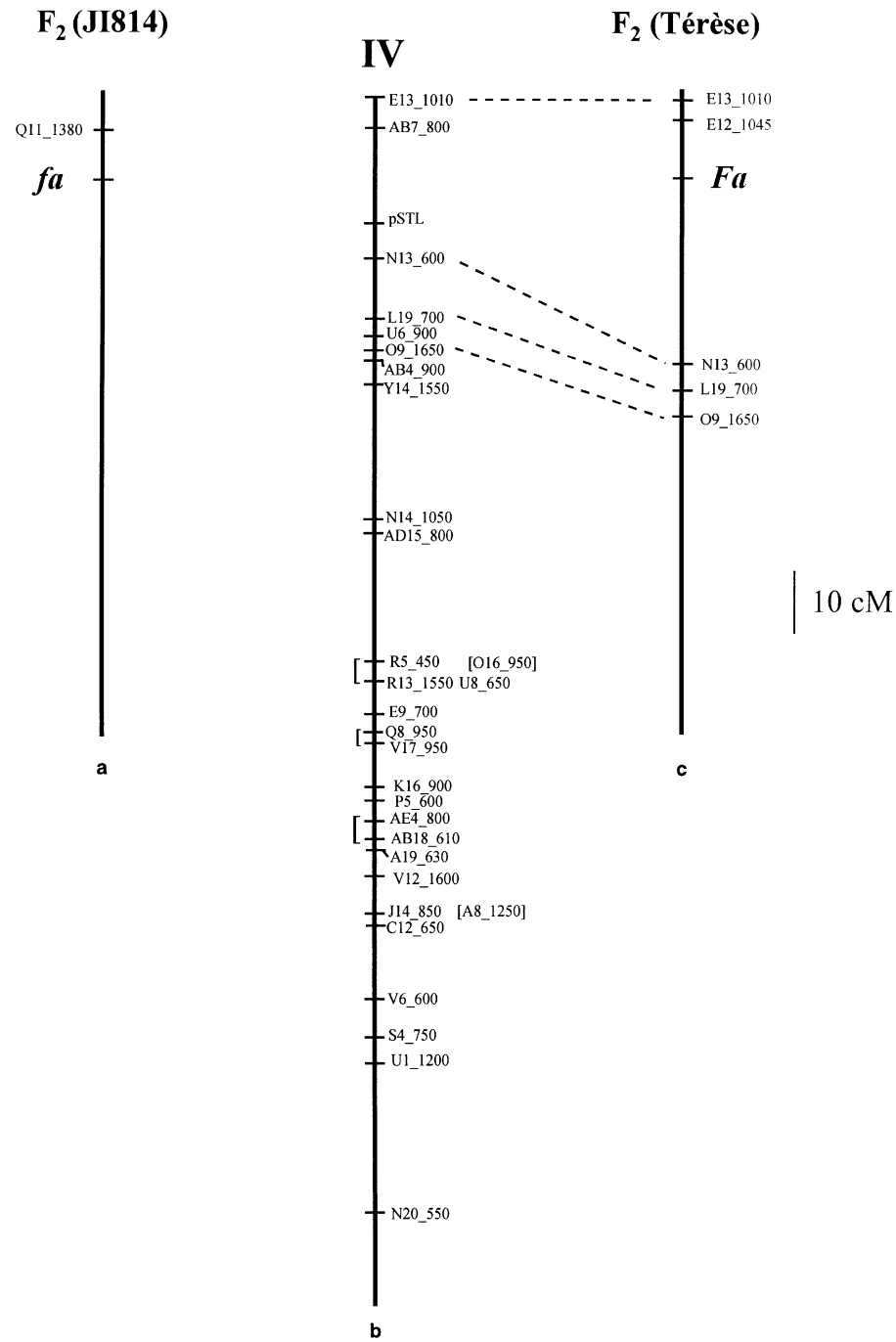


**Fig. 3 a–c** Linkage maps of the region surrounding the gene *Fa* derived from analysis of the $F_2$ population Térèse × JI814. **a** Marker specific to JI814. **c** Markers specific to Térèse. **b** Linkage map of linkage group IV derived from analysis of the RILs population, from Laucou et al. (1998). Groups of markers with a *square bracket* to the left cannot be ordered. *Markers between brackets* have been excluded on the basis of the three-point linkage data and were therefore placed approximately

*Dne* locus (Figs. 4 and 5)

Mutant $F_2$ segregants were visually distinct under short days, and clear separation is apparent from the two-way plot of flowering time against flowering node (Fig. 4). All early flowering $F_2$ segregants bred true in the $F_3$. Figure 4 shows the slight, but significant, delay in flowering time of the dwarf plants (*le*) in comparison with the tall plants (*Le*). The $F_1$ plants were late flowering like Térèse. The observed $F_2$ numbers of 16 *Dne Dne*, 42 *Dne dne* and 27 *dne dne* (with 23 missing data) fit a 1:2:1 ratio ($\chi^2 = 2.87$). The missing results are mainly due to the fact that some $F_2$ plants did not yield enough $F_3$ seeds to allow an unambiguous distinction between *Dne Dne* and *Dne dne* plants. RAPD analysis was done on the 108 plants and 15 homozygous day neutral plants and 17 homozygous photoperiodic $F_2$ plants were selected for preparation of the two pools.

The gene *Dne* was the first to be mapped in this study with the strategy of bulk segregant analysis, well before our RAPD map was achieved and before polymorphic bands between Térèse and Torsdag were identified.

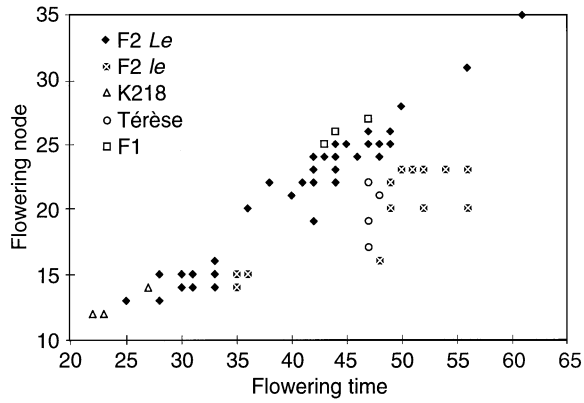


**Fig. 4** Flowering node plotted against number of days to first open flower (*flowering time*) for mutant line K218 (*dne*), Térèse, and the $F_1$ and $F_2$ plants of cross K218 × Térèse. K218 is tall (*Le*) and Térèse dwarf (*le*)

In fact, all primers from kits A to AD (600 primers) were screened on the two bulks. Later, when the RAPD map was finished and showed nine linkage groups (Laucou et al. 1998), other markers were analysed for the $F_2$ population to complete the map and to enable a comparison between the distances in the different maps.

We identified ten RAPDs polymorphic between the two bulks that were confirmed to be linked to the *dne* locus on the different individual DNAs that constituted each of the two bulked DNA samples. These markers are underlined in Fig. 5. This shows the efficiency of the strategy of bulk segregant analysis even if some markers close to the gene (I16_740, X17_1500) were not found to be bulk-specific. Most of them showed quantitative differences between the bulks and were not kept during the screening.

The S2_1900 RAPD fragment, which mapped at 4.7 cM from *dne*, was converted to a sequence-characterised amplified region (SCAR) for greater robustness during PCR (Paran and Michelmore 1993). A specific pair of primers (24-mers) were determined which at 55°C amplified a 1400-bp fragment in both Térèse and K218. After digestion with *Cfo*I, two bands were observed in Térèse at 980 and 420 bp. In K218, the same two bands were present together with the non-digested 1400-bp fragment (Fig. 2 c). These results can be explained by the presence of a duplicated gene, one being mutated in K218 at the *Cfo*I restriction site. This SCAR marker is analysed as a dominant marker by the presence or absence of the 1400-bp fragment. The order of the markers and the distances between markers are quite well conserved between the $F_2$ maps and the RILs map (Fig. 5).

*Sn* locus (Fig. 6)

Two flowering genes, *lf* and *sn*, were segregating in the cross HL59 (*lf sn*) × Térèse (*Lf Sn*). Under short-day conditions, which favour *Sn Dne Ppd* activity, early photoperiodic segregants (*lf Sn*) are readily distinguishable from day neutral segregants (*sn*) by several features. In the present study, the flower/leaf relativity index, the number of reproductive nodes and the peduncle length were the most effective variables. In early photoperiodic plants, the flowers opened further below the apical bud (i.e. flower/leaf relativity was lower), and substantially more reproductive nodes were produced before growth was arrested. In contrast, it was very difficult to determine the genotype for *Lf*, particularly in day neutral (*sn*) plants. Consequently, we were not able to map the gene *Lf* with this population.

The segregation for *Sn* was 17 *sn sn*, 42 *Sn sn* and 35 *Sn Sn* with four missing data. These results do not fit a 1 : 2 : 1 ratio ($\chi^2 = 7.96$, $P < 0.02$). A similar deficiency of recessive *sn* $F_2$ plants has been reported previously (Murfet 1971, 1978) but no explanation has been given yet. When we started the work on *Sn*, we knew on which linkage group of our RAPD map it should be according to Laucou et al. (1998) and the data from Poole et al. (1993). Consequently, we first looked for polymorphism between HL59 and Térèse among the RAPD markers of this linkage group. Fourteen RAPD markers were analysed, four in repulsion and ten in coupling in the $F_2$ population. Segregation for these RAPD markers fitted the expected ratio (1 : 3), the highest $\chi^2$ value being 2.30 for AA7_1700, the closest marker at 8.4 cM from *Sn* (Fig. 6). Unfortunately, X1_420, O19_1200 and particularly L19_550, which is specific to Torsdag, were not polymorphic between Térèse and HL59. We were unable to map the co-dominant marker L13_1350/L13_1180 in this $F_2$ population because the presence of supplementary bands in HL59 made the reading of the interesting band difficult.

*Rms2* locus (Fig. 7)

Two bulks of DNA from 12 *Rms2* $F_2$ plants, that were either homozygous or heterozygous, and 12 *rms2* $F_2$ plants were compared. Bulk segregant analysis was first performed with RAPDs specific to Térèse which were scattered on the different linkage groups of the RAPD map. Four RAPDs located on linkage group IB (Z6_700, R11_730, N13_900, A4_430) were found to be bulk-specific and confirmed to be linked to *Rms2* by the analysis of the 199 $F_2$ plants. Other markers specific to Torsdag and located in this region on the RILs map (H14_1250, AD4_1000, E12_800) were also analysed. The resulting map is presented in Fig. 7. It was not possible to precisely locate *rms2* relative to AD4_1000 (Fig. 2 d) and E12_800 or to obtain a significant linkage between R11_730 and the group-IB marker *Af* which was also segregating in this cross, whereas in the RILs map the two-point distance between R11_730 and *Af* is 21.5 cM. The distances covered in this region

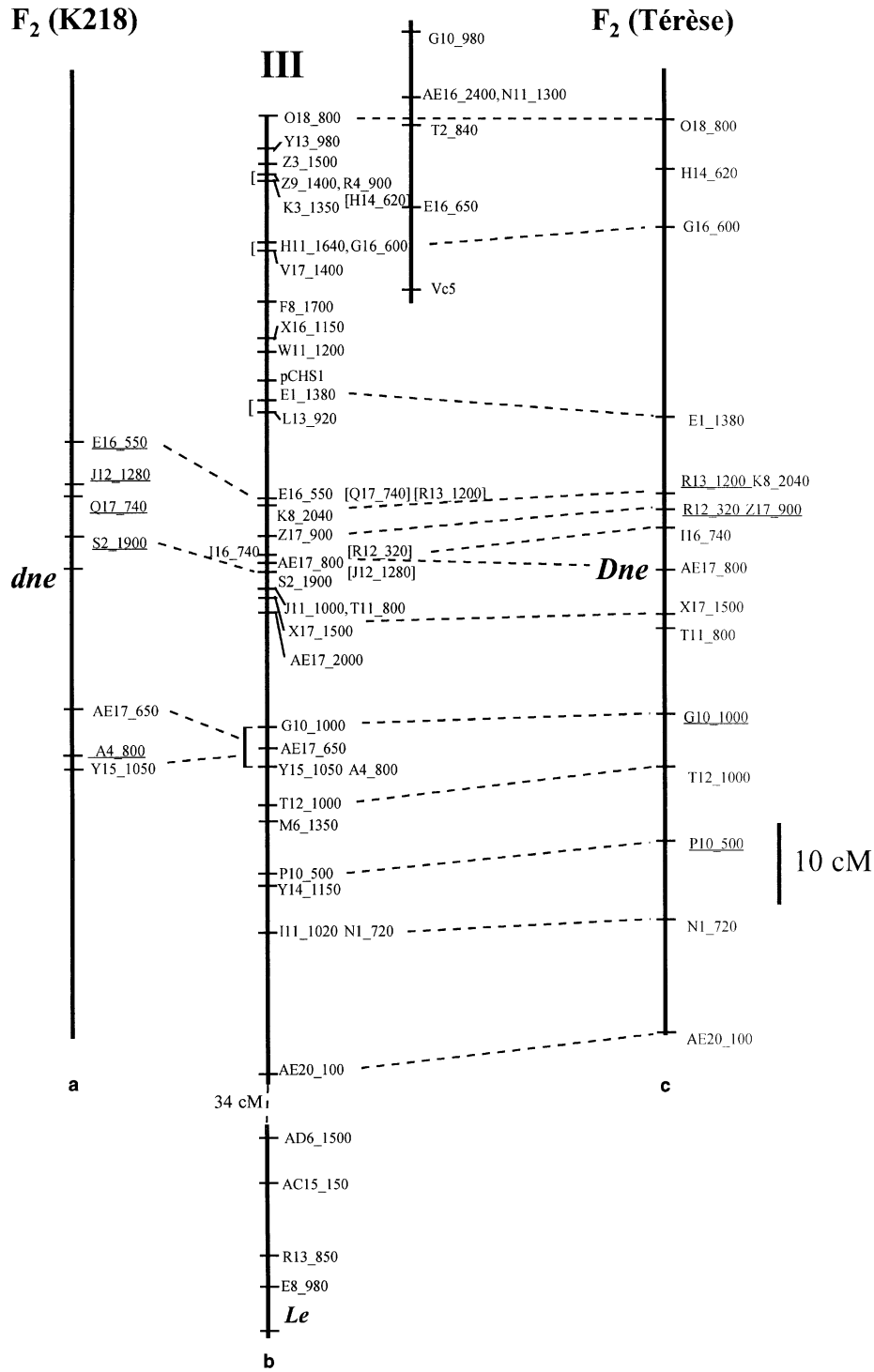


**Fig. 5 a–c** Linkage maps of the region surrounding the gene *Dne* derived from analysis of the $F_2$ population K218 × Térèse. **a** Markers specific to K218 (ex Torsdag). **c** Markers specific to Térèse. **b** Linkage map of linkage group III derived from analysis of the RILs population, from Laucou et al. (1998). Groups of markers with *a square bracket* to the left cannot be ordered. *Markers between brackets* have been excluded on the basis of the three-point linkage data and therefore were placed approximately

by the RAPDs specific to Térèse are slightly increased in the $F_2$ map in comparison with the RILs map.

*Rms3* locus (Fig. 8)

The first cross used to map *Rms3* was WL6042 × Térèse. The segregation for *Rms3* fitted a 1:2:1 ratio (12:32:13, $\chi^2 = 0.90$). Two bulks of DNAs from nine $F_2$ mutant plants and seven *Rms3 Rms3* plants were compared. Of the 380 primers tested, only two, V20_1100 and H7_800, were bulk-specific (and WL6042-specific) and could be analysed for the 57 individuals of the $F_2$ population.

The V20_1100 RAPD fragment was cloned and sequenced to derive a SCAR. The identity of the cloned

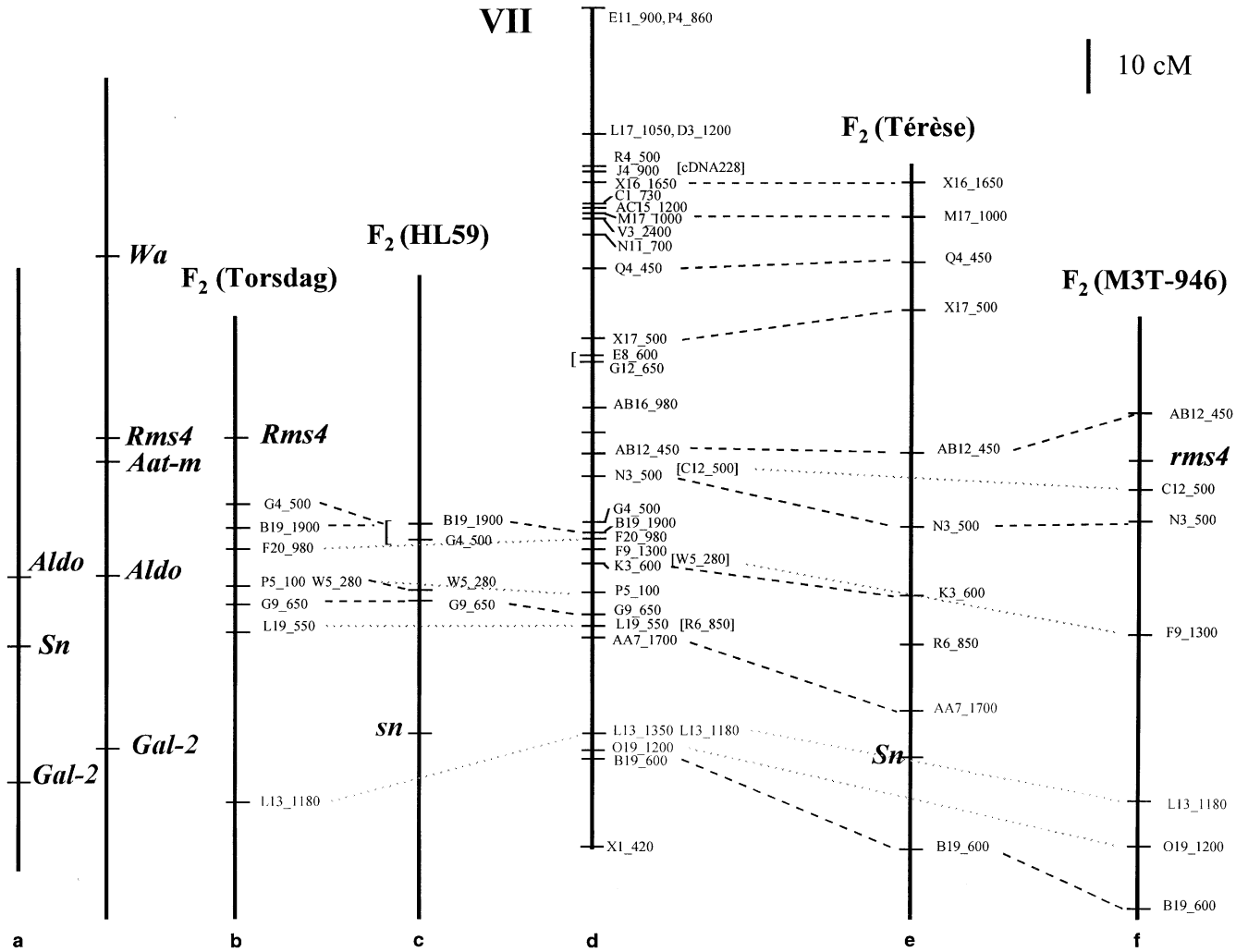


**Fig. 6 a** Classical linkage maps surrounding the genes *Rms4* (Poole et al. 1993) and *Sn* (Murfet and Sherriff 1996). **b** and **f** Linkage maps of the region surrounding the gene *Rms4* derived from analysis of the $F_2$ population M3T-946 × Torsdag. **b** Markers specific to Torsdag. **f** Markers specific to M3T-946 (ex Térèse). **c** and **e** Linkage maps of the region surrounding the gene *Sn* derived from analysis of the $F_2$ population HL59 × Térèse. **c** Markers specific to HL59. **e** Markers specific to Térèse. **d** Linkage map of linkage group VII derived from analysis of the RILs population, from Laucou et al. (1998). Groups of markers with a *square bracket* to the left cannot be ordered. *Markers between brackets* have been excluded on the basis of the three-point linkage data and therefore were placed approximately.

product was confirmed by hybridization of the cloned fragment to Southern blots of 32 $F_2$ individuals of the cross WL6042 × Térèse that segregated for V20_1100. The hybridization pattern was identical to the RAPD segregation. After sequencing, a pair of 24-mer primers was designed. A single band of 900 bp was amplified in both parents at an annealing temperature of 55°C. Digestion with *Hin*fI revealed a fragment length polymorphism (Fig. 2 e). After digestion, the amplified fragment from WL6042 gave a strong band at 480 bp whereas the amplified fragment from Térèse gave a band at 520 bp. In heterozygous plants, another strong band of approximately 700 bp was observed. This band can be explained by the formation of a heteroduplex molecule with two allelic sequences of different size and with a reduced electrophoretic mobility (Ayliffe et al. 1994). We have confirmed this hypothesis by mixing the two amplified fragments from Térèse and WL6042 followed by a single PCR amplification cycle in the absence of *Taq* DNA polymerase, and digestion. After gel electrophoresis, a third band with a reduced electrophoretic mobility was apparent. The SCAR V20_1100 could be scored as a co-dominant marker and mapped at 16.7 cM from *Rms3*.

Mutant T2-30 recently obtained from Térèse has proven to be a new allele of *rms3* (Rameau et al. 1997). We therefore used the cross T2-30 × Torsdag to complete the region with other markers. The $F_2$ segregation for *Rms3* fitted a 1:2:1 ratio (46:85:46, $\chi^2 = 0.28$). The SCAR V20_1100 and RAPD markers close to V20 were analysed for the $F_2$ population. All fitted the expected ratio of 1:2:1 for the SCAR V20 (44:73:46) and 3:1 for the RAPDs.

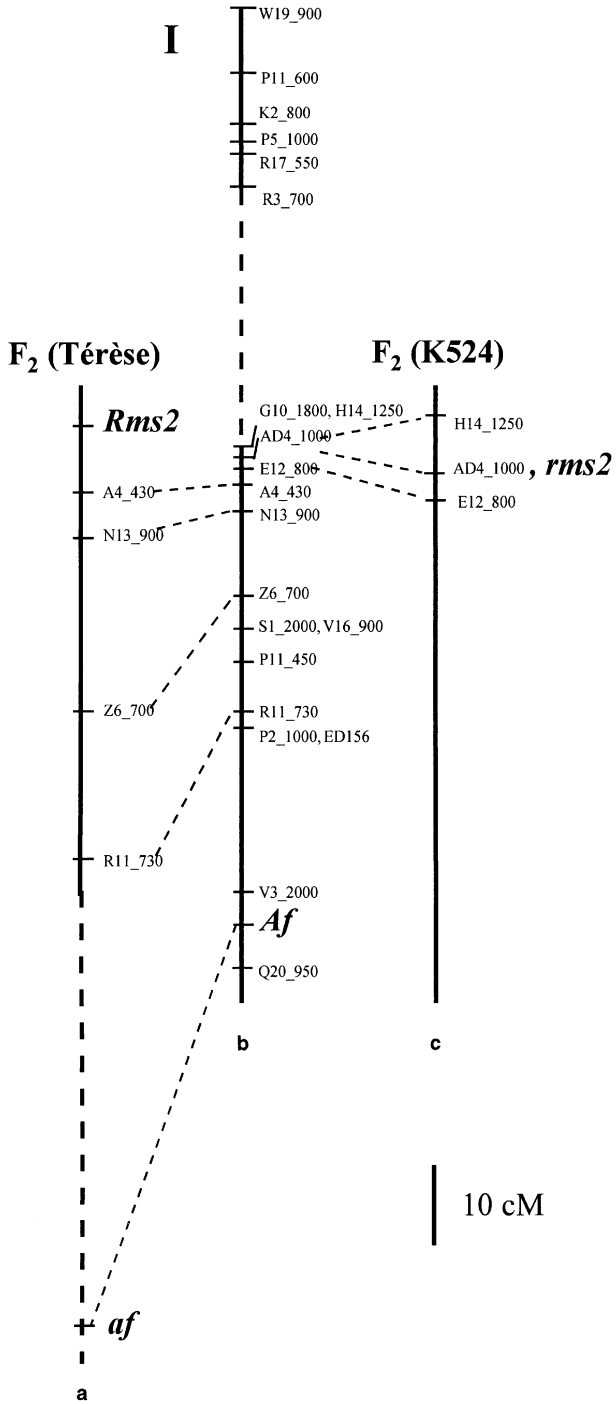


**Fig. 7 a–c** Linkage maps of the region surrounding the gene *Rms2* derived from analysis of the $F_2$ population K524 × Térèse. **a** Markers specific to Térèse. **c** Markers specific to K524 (ex Torsdag). **b** Linkage map of linkage group I derived from analysis of the RILs population, from Laucou et al. (1998). Groups of markers with a *square bracket* to the left cannot be ordered. *Markers between brackets* have been excluded on the basis of the three-point linkage data and therefore were placed approximately

Recently, it has been shown that the nodulin gene, *Enod2*, is linked to markers on both linkage group IA and linkage group II (Paruvangada et al. 1995) and the two groups have been combined into a single group in the pea map of Weeden et al. (1996) and in the revised version (Hall et al. 1997) of the map from Ellis et al. (1992). We tried to connect *Rms3* with unlinked markers or markers located at the end of linkage groups by comparing the profiles of 11 *rms3 rms3* plants and 11 *Rms3 Rms3* plants. By this method, one of the two previously unlinked markers, T3_650, was found to be rather specific to *rms3 rms3* plants and mapped at 9.5 cM from *Rms3*.

For the markers in repulsion with *Rms3* (specific to T2-30), the order and the distances are quite well conserved with the map obtained on the RILs population (Fig. 8). The data placed the *Rms3* locus toward the end of linkage group IA-II (currently linkage group II), the closest marker being T3_650. This position was subsequently confirmed from the $F_2$ of the cross SGE80 × K564 which segregated for *rms3*, SCAR V20_1100, the isozyme marker *Aat-p* and the anthocyanin gene *A* (Fig. 8 e).

*Rms4* locus (Fig. 6)

The strategy of bulk segregant analysis was not necessary to find linked markers to *Rms4* as we knew approximately its position from Laucou et al. (1998) and the data of Poole et al. (1993), and the position of *Sn*. Segregation for *Rms4* was not significantly different from a 1:2:1 ratio (59:91:44, $\chi^2 = 3.06$). Fifteen RAPDs were analysed for the 197 $F_2$ plants. All fitted the expected 1:3 ratio except for O19_1200 (63:130, $\chi^2 = 6.00$). The co-dominant RAPD marker L13_1180 fitted a 1:2:1 ratio (50:85:58, $\chi^2 = 3.40$). The map distance covered by the linkage group between markers B19_600 and AB12_450 was slightly increased when we added the marker O19_1200 (93.8 cM vs 85.8 cM). The distance between the same two markers is greater than the distance covered by the linkage map obtained from the RILs (57.1 cM), but the order of the markers is conserved. Two easy to score markers, AB12_450 and C12_500 (Fig. 2 g), have been found flanking the *rms4* gene at 7.9 and 5.3 cM, respectively.

## Conclusion

RAPD markers have been commonly used in genetic studies because they allow the quick construction of genetic maps and, combined with the strategy of bulk segregant analysis, are particularly adapted to find linked markers to a gene of interest in a short time. For seven genes which have a marked effect on the global architecture of the plant, and in particular on

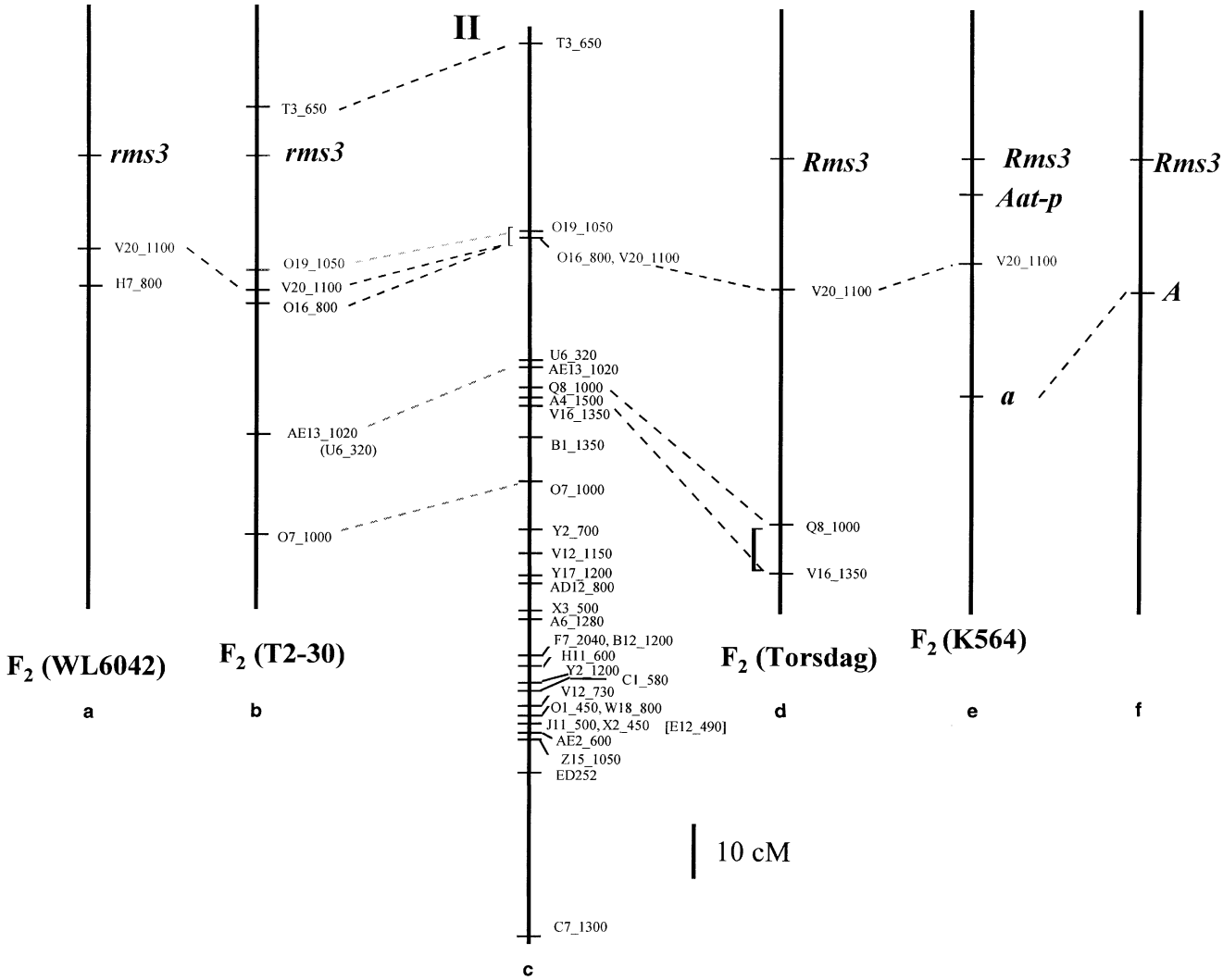


**Fig. 8** **a** Linkage map of the region surrounding the gene *Rms3* derived from analysis of the $F_2$ population WL6042 × Térèse. **b** and **d** Linkage maps of the region surrounding the gene *Rms3* derived from analysis of the $F_2$ population T2-30 × Torsdag. **b** Markers specific to T2-30 (ex Térèse). **d** Markers specific to Torsdag. **c** Linkage map of linkage group II derived from analysis of the RILs population, from Laucou et al. (1998). **e** Linkage map of the region surrounding the gene *Rms3* derived from analysis of the $F_2$ population SGE80 × K564. **f** Classical linkage map surrounding the gene *Rms3*, from Poole et al. (1993). Groups of markers with a *square bracket* to the left cannot be ordered. *Markers between brackets* have been excluded on the basis of the three-point linkage data and therefore were placed approximately

branching, we have established a precise genetic map of the region containing these genes which are located on six of the current seven pea linkage groups. Generally, RAPDs have been easily used as common markers between crosses even if Térèse and/or Torsdag were not both involved.

For most of these genes, closely linked markers were found. Three RAPD bands, U16_1300, Q20_1100 and U8_1900, in repulsion with the gene *Det*, mapped at less than 2 cM from the gene. For the flowering gene *Dne*, the region surrounding the gene is quite well saturated and the SCAR S2_1900 mapped at 4.7 cM from the gene while the other marker in repulsion (specific to the mutant K218), and on the other side of the gene AE17_650, mapped at 17.4 cM. These markers are currently being used to facilitate the introgression of *dne* in breeding materials, in particular in the Térèse background. Unfortunately, we were not able to find closely linked markers to the gene *Sn*, the closest, AA7_1700, being in coupling and located at 8.4 cM from *Sn*. There was also a shortage of markers for *Fa* and *Rms3*.

For four of the seven mapped genes, $F_2$ populations were developed from crosses involving the Torsdag and/or Térèse background (Table 1). In such crosses, polymorphic RAPD markers were already known and ordered into nine linkage groups (Laucou et al. 1998). With this system, it was particularly easy to locate *Rms2*, which was not mapped previously. Generally, there were no significant differences in gene order or

rate of recombination between different crosses, and combining the RILs map and the two $F_2$ maps should not be a problem especially when the cross used involves isogenic lines of Térèse and Torsdag. In that case, combining the maps makes sense, as the common markers have the same position in the original maps and no difference in linkage arrangements between crosses is expected. In this context, Térèse, Torsdag and their isogenic lines derived from mutagenesis programs have the same advantages as multiple-marker lines.

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