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Restriction fragment length polymorphism analysis of loci associated with disease resistance genes and developmental traits in *Pisum sativum* L.

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Abstract An F_2 population of pea (*Pisum sativum* L.) consisting of 174 plants was analysed by restriction fragment length polymorphism (RFLP) and random amplified polymorphic DNA (RAPD) techniques. *Ascochyta pisi* race C resistance, plant height, flowering earliness and number of nodes were measured in order to map the genes responsible for their variation. We have constructed a partial linkage map including 3 morphological character genes, 4 disease resistance genes, 56 RFLP loci, 4 microsatellite loci and 2 RAPD loci. Molecular markers linked to each resistance gene were found: Fusarium wilt (6 cM from *Fw*), powdery mildew (11 cM from *er*) and pea common Mosaic virus (15 cM from *mo*). QTLs (quantitative traits loci) for *Ascochyta pisi* race C resistance were mapped, with most of the variation explained by only three chromosomal regions. The QTL with the largest effect, on chromosome 4, was also mapped using a qualitative, Mendelian approach. Another QTL displayed a transgressive segregation, i.e. the parental line that was susceptible to Ascochyta blight had a resistance allele at this QTL. Analysis of correlations between developmental traits in terms of QTL effects and positions suggested a common genetic control of the number of nodes and earliness, and a loose relationship between these traits and height.

Key words Restriction fragment length polymorphism (RFLP) · *Pisum sativum* L. · Disease resistances · Developmental traits · Quantitative trait loci (QTL)

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Introduction

Ascochyta blight, Fusarium wilt, powdery mildew and pea common Mosaic virus are four important diseases of pea (*Pisum sativum* L.), and they are responsible for economic losses in most of the temperate pea-growing regions in the world. Variation in yield is often associated with these epidemic diseases.

Resistance to *Ascochyta pisi* race C, the most aggressive and widespread pathotype in Europe, is controlled by several genes, but the inheritance and the number of genes involved have not been precisely determined. Cousin (1974) suggested the existence of a major gene with intermediate dominance and several minor genes. Darby et al. (1985) reported the existence of a dominant major gene, *Rap 2*, and of a minor gene or polygenic system that modifies the action of *Rap 2*. *Rap 2* has been located on chromosome 1, loosely linked to the *i* locus. Such results led us to use a quantitative trait loci (QTL) mapping approach to analyse the genetic determinism of *Ascochyta pisi* race C resistance.

Resistance to *Fusarium oxysporum* f. sp. *pisi* race 1, *Erysiphe polygoni* and pea common Mosaic virus are controlled by single genes (*Fw*, *er* and *mo*, respectively). All of these genes have already been assigned to choromosomes: *Fw* was reported to be on chromosome 4 (Wells et al. 1949), *mo* on chromosome 2 (Marx and Provvidenti 1979) and *er* was first reported to be on chromosome 3 (Harland 1948), and more recently on chromosome 6 (Wolko and Weeden 1991).

Our approach was to characterize an F_2 population by morphological, restriction fragment length polymorphism (RFLP) and random amplified polymorphic DNA (RAPD) markers, and to score F_2 plants or their progeny for resistance or susceptibility to each pathogen. A partial linkage map including 3 morphological character genes, 4 disease resistance genes, 56 RFLP loci, 4 microsatellite loci and 2 RAPD markers is presented here. Molecular markers for each type of

monogenic resistance were identified, and QTLs affecting *Ascochyta pisi* race C resistance, plant size, earliness of flowering and node number were detected. The QTLs allowed us to account for most of the variation for the traits examined except height and were consistent with the correlations observed between some characters.

Materials and methods

Plant material

For RFLP and RAPD mapping, a population of 174 F_2 plants derived from a cross between two pea homozygous varieties, 'Erygel' and '661', was used. 'Erygel' was obtained by the Institut National de la Recherche Agronomique (INRA, France) in progeny of the cross between 'Mexique 4' (INRA), which is resistant to powdery mildew, and 'Lancet' (Van Waveren, FRG), which is resistant to pea common Mosaic virus. '661' (INRA) is derived from a cross between 'Finale' (Cebeco, the Netherland), which is resistant to *Ascochyta pisi* race C and *Fusarium oxysporum* race 1, and 'Wis 7105 × 7006' (University of Wisconsin, USA) an *afila* line (*afila* genotypes have tendrils in place of leaflets). 'Erygel' and '661' differ for various characters: 'Erygel' is semi-dwarf and semi-early (first fertile node: 12th–13th nodes), has wrinkled (*r/r*) and yellow (*I/I*) seeds and normal leaflets (*Af/Af*), is susceptible to Fusarium wilt race 1 (*fw/fw*) and to *Ascochyta pisi* race 1 and is resistant to powdery mildew (*er/er*) and to pea common Mosaic virus (*mo/mo*); '661' is dwarf and late (first fertile node: 14th–15th nodes), has smooth (*R/R*) and green (*i/i*) seeds and afila leaflets (*af/af*), is resistant to Fusarium wilt race 1 (*Fw/Fw*) and to *Ascochyta pisi* race 1 and is susceptible to powdery mildew (*Er/Er*) and to pea common Mosaic virus (*Mo/Mo*). Most of the genes responsible for these characters have been located: *i* (Lamprecht 1948) and *af* (Khangildin 1966) are on chromosome 1, *mo* is on chromosome 2 (Marx and Provvidenti 1979), *Fw* is on chromosome 4 (Wells et al. 1949), *r* is on chromosome 5 (Weeden and Wolko 1990) and *er* is on chromosome 6 (Wolko and Weeden 1991).

From a total of 174 F_2 plants, 72 were grown in the greenhouse and 102 in the field. All F_2 plants were self-pollinated to obtain F_3 families that were used for some of the resistance tests (see below). Since there were less than 30 F_3 seeds for the greenhouse F_2 plants, a second multiplication step was needed. For these genotypes the resistance tests were done on 20 F_4 plants (5 F_4 plants per F_3 plant × 4 F_3 plants) to determine the genotype of each F_2 plant. Four F_3 plants to infer the F_2 genotype results in an error risk of only 0.02 (the possible error concerns heterozygous F_2 plants scored as having the homozygous genotype).

Developmental traits

The number of nodes from the bottom to the first fertile node was noted at maturity on the 102 F_2 plants grown in field. For the F_2 plants grown in the greenhouse, the number of nodes and the height were noted at maturity, and the earliness of flowering was determined by observation of the plants every 4 days for 16 days (score from 1 to 5).

Pathological tests

Fusarium oxysporum f.sp pisi race 1 resistance test

The pathogenic isolate was maintained on Mathur (1950) medium, which consists of (per liter): 2 g pancreatic peptone, 2.8 g glucose, 1.23 g magnesium sulphate, 2.72 g potassium dihydrogenophosphate, 10 mg yeast extract, 20 g gelose. The inoculum was prepared according to Hubbeling (1956). Fungi were grown at 24 °C in Czapek-Dox liquid medium (2 g sodium nitrate, 1 g dipotassium phosphate, 0.5 g magnesium sulphate, 0.5 g potassium chloride, 30 g glucose for 1 liter of distilled water; the solution is sterilized at 115 °C for 20 min). The liquid culture was continuously aerated by sterile air. After 5 or 6 days of culture at 25 °C, the inoculum was mixed with sand. The pea seeds were sown and then covered with 1 cm of infected sand. The 'Rondvil' variety (Vilmorin, France) was used as the susceptible control. Plants were grown in the greenhouse at 21 °C under high humidity and supplementary lighting to provide a 16-h photoperiod. Observation of the seedlings were made 3 weeks after inoculation. Susceptible seedlings were smaller than the resistant ones and their leaves were green-grey. Susceptibility was noted again 7 days later: susceptible seedlings wilted and died. The F_2 genotypes were determined from observation of their F_3 or F_4 progeny, according to the site of the F_2 plants (field or greenhouse, respectively – see Plant material section).

Pea common Mosaic virus resistance test

The virus was maintained on susceptible pea plants. Young infected leaves showing clear symptoms were crushed in a mortar. This inoculum was used immediately to avoid virus degration. Plants were grown in a greenhouse at 20 °C under a 14- to 16-h daylength. At the two- or three-leaf stage the isolate was added to the plant following soft abrasion with Carborundum powder. Care was taken to avoid excessive damage of the tissue to prevent necrosis. Two stipules or leaflets per plant were infected. Observation of the seedlings were made 15 days after inoculation. Susceptible plants show vein clearing and mosaic patterns on the leaf. As for Fusarium wilt, the F_2 genotypes were determined from observation of their F_3 or F_4 progeny, according to the site of the F_2 plants.

Powdery mildew resistance test

Since *Erysiphe polygoni* f.sp *pisi* is naturally present in fields, infection occurs from natural sources. '661' and 'Erygel' lines were used as the susceptible and resistant controls, respectively. Infected foliage surfaces were white and powdery. Tissue beneath the infected areas sometimes turned purplish followed by the production of black fruiting structures, infected tissue remained soft and failed to dry out naturally and the maturity of susceptible plants was delayed. In resistant plants infection was absent or localized in very small patches. The observations were made in the field on F_2 plants. Due to the dominance of susceptibility over resistance, the genotypes *Er/Er* and *Er/er* were indistinguishable.

Ascochyta blight resistance test

A pathogenic isolate of *Ascochyta pisi* race C was maintained and multiplied on Mathur medium. Pea seeds were sown in sand and placed at 20 °C under high humidity. The 'Kelvedon Wonder' variety (Hurst, UK) was used as the susceptible control. Ten days after sowing, the seedlings were inoculated by mist spraying a suspension of spores from a 3- or 4-day-old culture two or three times per day for 15 min. Ascochyta blight symptoms appeared 10 days after the inoculation. The seedlings were scored for resistance one week after the first symptoms. Plants were given a score, ranging from 0 (for the most resistant) to 5 (for the most susceptible). The classes used were as follows: 0 (no symptom), 1 (few necrotic points on the stem), 2 (necrotic spots of small size), 3 (many necrotic spots), 4 (stem surrounded with necrotic region), 5 (death of the plant). Qualitative analysis was performed to localize a putative major resistance gene, and quantitative analysis to detect other genes involved in the resistance of *Ascochyta pisi*. For qualitative analysis, the F_2 plants were classified into three categories, i.e. resistant (where all the progeny were noted as 0 or 1), susceptible (progeny noted as 2–5) or segregating (where the progeny were composed of resistant and susceptible plants). For quantitative analysis, the mean of the F_3 was attributed to the parental F_2 plant.

Nucleic acid manipulation

Clones to be used as probes in RFLP analysis were selected from a partial *Pst*I library of genomic pea DNA. Pea DNA (300 µg) was digested with

*Pst*I and then fractionated on a 1% agarose gel. The area of the gel containing fragments in the region of 0.5–2 kb was excised, and the DNA was extracted by electroelution. The isolated DNA fragments were ligated into *Pst*I-cut M13 mp18, the ligation mix was transformed into JM 101 and the recombinant phages were selected using IPTG and Xgal (blue and white) selection. A total of 166 *Pst*I genomic probes were tested for polymorphism with 'Erygel' and '661' genomic DNAs digested with *Eco*RI and *Hin*dIII.

The isolation of genomic DNA was performed as described by Burr et al. (1988). Approximately 7 µg of genomic DNA was digested using *Eco*RI or *Hin*dIII. The DNA was digested with 20 U of enzyme according to the manufacturer's instructions for 4 h in the presence of 2.5 m*M* spermidine and then loaded onto a 0.8% (w/v) agarose gel (25 cm × 20 cm) in the presence of 1 × TPE buffer (40 m*M* Tris-phosphate, pH 8.0, 1 m*M* EDTA) and electrophoresed overnight (30 V). Depurination and vacuum transfer (2 h, 50 mbar) of the DNA from the gel onto a nylon membrane was performed as described by Olszewska and Jones (1988).

The inserts of the genomic *Pst*I probes were amplified by the polymerase chain reaction (PCR). Purified DNA inserts (50–250 ng) were labeled with 3000 µCi mmol^{-1} α-[^{32}P]d CTP using the random primer labeling procedure of Feinberg and Vogelstein (1983).

Prehybridization, hybridization and washing of the blots were as described by Church and Gilbert (1984). The membranes were exposed to X-ray film (Kodak, X-OMAT-AR) with one intensifying screen at −76 °C for 7 days. For fingerprinting, the oligonucleotide $(GAA)_6$, kindly provided by JT Epplen (Max Planck Institut für Psychiatrie, Martinsried, FRG), was used. Genomic DNA was digested with *Hin*dIII, *Hin*fI or *Dra*I. Hybridization on dried gels was performed according to Schäfer et al. (1988). In order to reprobe the gels, the probes were removed by incubating the gels in 0.5 *M* NaOH, 0.15 *M* NaCl for 30 min, then in 0.5 *M* Tris, 0.15 *M* NaCl, pH 8, for 30 min, and were equilibrated in 6 × SSC for 20 min at room temperature.

For RAPD analysis, the procedure described by Williams et al. (1990) was used. Forty primers from Operon (Alameda, Calif.) were tested for polymorphism between 'Erygel' and '661', with 200 ng of genomic DNA being used per sample. The samples were first held at 94 °C for 3 mn and then were subjected to 45 PCR cycles of melting at 95 °C for 20 s, annealing at 35 °C for 1 mn and extending at 73 °C for 2 mn. The products were separated on 1.8% agarose gels, and the gels were subsequently stained with ethidium bromide to allow the DNA to be visualized.

Linkage analysis and QTL mapping

Linkage analysis of the F_2 population was performed on a Vax workstation using the MAPMAKER software (Lander et al. 1987). All possible pairwise comparisons were made between marker loci, and recombination fractions were calculated. The markers were then divided into linkage groups based on pairwise associations. Finally, three-point and n-point analyses were performed to determine the most likely order for markers. Map distances (centiMorgan, cM) were then estimated using recombination fractions and Kosambi's mapping function (Kosambi 1944) between ordered marker loci. The linkage map was established using all 174 F_2 plants. The linkage groups were obtained by choosing 0.3 as the maximum recombinant fraction and 3 as the minimum LOD score value. The confidence intervals of the distances ($\alpha = 0.05$) were computed using Allard's formulae (1956) according to the different modes of inheritance at the marker loci: codominance/codominance, codominance/dominance and dominance/dominance.

For QTL detection, greenhouse and field plants were analysed separately. The data were analysed with a programme using the SAS GLM procedure (SAS Institute 1982). One-way ANOVAs were performed with the marker locus as the factor and the three genotypes as levels. Significant *F*- values were interpreted to indicate segregation of a QTL linked to the marker locus. Due to the large number of ANOVAs performed, we chose a type-I error of 0.01 to minimize the number of false positive QTLs. The variation attributed to each marker locus was considered to be a proportion of the total variation for each trait, and this proportion was recorded as an R^2 value [R^2 = SS (marker)/total SS; SS is for sum of squares]. Additive and dominance effects attributable to QTLs were determined from differences between mean trait values of marker locus genotypic classes. Student-tests were used to examine the significance of dominance effects by comparing the heterozygote class to the mean of the homozygote classes. The ratio (d/a) of the estimated dominance effect over the estimated additive effect was used to measure the degree of dominance. Results were confirmed by using MAPMAKER-QTL software with a LOD score threshold of 2.2. This value was chosen from the theoretical considerations of Lander and Botstein (1989), given that pea has seven chromosomes, a possible genome length of 1700 cM (Ellis et al. 1992) and that we had a mean spacing between markers of about 20–25 cM.

Results

Inheritance of disease resistances

The segregation data for the disease resistance genes observed in the F_2 plants or their F_3 or F_4 progeny derived from a cross between the pea varieties 'Erygel' and '661' are listed in Table 1. Segregations of Fusarium wilt and Ascochyta blight resistances fit the expected 1:2:1 ratio for single genes. This result confirms that *Ascochyta pisi* race C resistance may be controlled by a major gene. For *mo*, a significant deviation from the 1:2:1 ratio was observed, with an excess of heterozygotes ($P < 0.05$), and segregation of *er* fit the expected 1:3 ratio for a single recessive resistance gene.

RFLP analysis

When a single enzyme was used, either *Eco*RI or *Hin*dIII, 35% of the 166 probes revealed DNA polymorphism between 'Erygel' and '661'; when both *Eco*RI and *Hin*dIII were taken together, 50% of the probes revealed polymorphism. Among the 166 probes, 40 revealed polymorphism with both *Eco*RI and *Hin*dIII. The abilities of the two enzymes to reveal polymorphisms were not independent ($\chi^2 = 34.5$; $P < 0.001$).

The 50 probes that gave the most clearly readable patterns were selected: 26 were hybridized on F_2 DNAs digested with *Eco*RI and 24 were hybridized on F_2 DNAs digested with *Hin*dIII. Nearly all of the probes gave a pattern with more than 4 bands. For 17 probes the patterns observed in the two parents differed only for 1 band; for 27 probes the patterns differed for 2 bands, one specific for 'Erygel' and the other specific for '661'. In addition, all of the F_2 plants had at least 1 of the 2 bands, and the segregations were according to the 1:2:1 ratio. In such cases, the 2 bands were considered to be alleles of a single locus. For the remaining 6 probes, the patterns of the two parents differed for several bands. For

Table 1 Segregation and chi-square goodness-of-fit analyses for disease resistances measured on F_3 or F_4 families (Fusarium wilt, pea common Mosaic and *Ascochyta pisi* race C: segregation 1:2:1) or on F_2 plants (powdery mildew: segregation 1:3)

Disease resistance gene	Observed ratio	χ^2 1:2:1	χ^2 1:3
fw	41:69:37	0.77	–
mo	21:85:28	6.44*	–
Ascochyta pisi race C	30:65:37	2.19	–
er	24:73	–	0.003

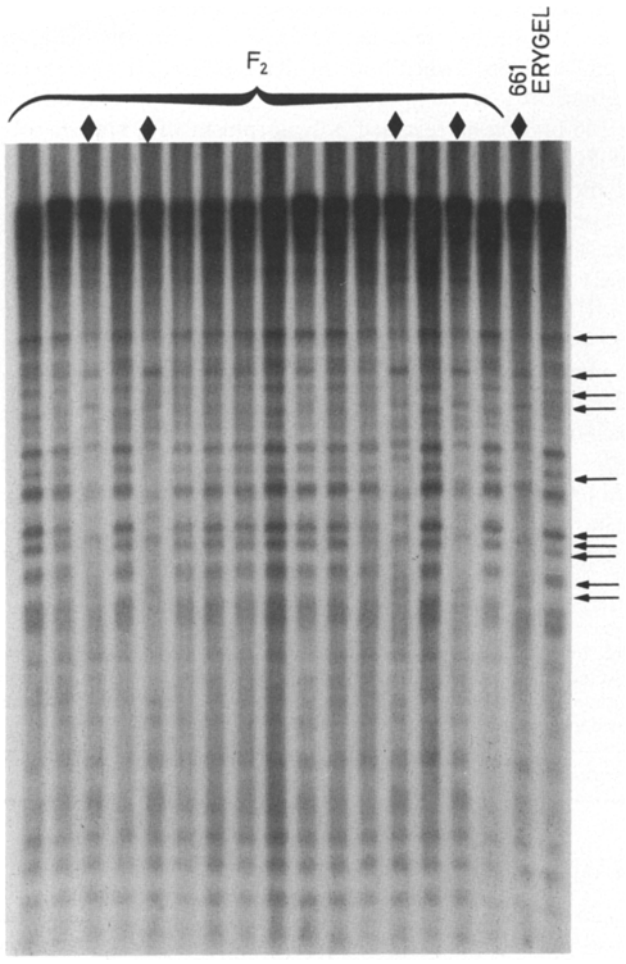
* Indicates significant ($P < 0.05$) deviation from the expected segregation

these probes the analysis of the pattern of the F_2 plants was performed considering single bands as dominant markers and the absence of a band as the alternative allele. Allelism tests were also done to reveal whether different bands were alleles of the same RFLP locus. For example, in the case of probe p309, 5 bands were analysed independently and 3 distinct loci were identified: p309-1, 5, p309-2, 3, p309-4. Thus, a total of 58 RFLP loci were detected from the 50 selected probes.

The $(GAA)_6$ oligonucleotide probe revealed numerous polymorphic bands between 'Erygel' and '661' for every enzyme used. Eleven polymorphic bands from a *Dra*I digest were scored independently and noted (GAA)-1 to (GAA)-11. As previously, the allelism of the different bands was tested. Five loci were identified: (GAA)-1, 2, 3, 4, 6, 8, (GAA)-5, (GAA)-7, (GAA)-9, (GAA)-10, 11. Only two patterns, which differ for 10 bands, were identified with *Hin*fI among the F_2 progeny: the '661' pattern and the 'Erygel' one, with the latter being indistinguishable from the pattern of the F_1 hybrid (Fig. 1). This locus was denoted (GAA)-12. Three patterns were observed with *Hin*dIII in the F_2 progeny: 'Erygel', '661' and F_1 hybrid types. The locus was denoted (GAA)-13. A comparison of the *Hin*fI and *Hin*dIII patterns showed that the same locus was identified and was termed (GAA)-12, 13. Hence a total of 6 loci were identified with the oligonucleotide $(GAA)_6$.

For RAPD analysis, 18 out of 40 primers revealed a polymorphism, 5 of the patterns being easily readable. One of those was used to analyse the F_2 progeny, allowing the amplification of a fragment specific from 'Erygel' and another from '661'.

Fig. 1 DNA fingerprints of F_2 plants generated using *Hin*fI for digestion and $(GAA)_6$ for hybridization. Two patterns were noted in the progeny: that of '661', indicated by a *black symbol* (◆), and that of 'Erygel', which is indistinguishable from the F_1 hybrid pattern. The *arrows* point to bands that differ between the two patterns



Genetic map

The genetic linkage map consisted of 12 linkage groups covering 550 cM (Fig. 2). This map includes 3 morphological character genes, 4 disease resistance genes, 56 RFLP loci, 4 microsatellite loci and 2 RAPD loci. Two RFLP loci (p62, p286) and 2 microsatellite loci, (GAA)-5 and (GAA)-10, 11, remain independent. Five linkage groups could be assigned to chromosomes using the previously published locations of morphological and disease resistance genes (see Materials and Methods): *af* and *i* to chromosome 1, *mo* to chromosome 2, *Fw* to chromosome 4, *r* to chromosome 5 and *er* to chromosome 6. For each resistance gene, linked RFLPs were found: p252 is 15.9 ($\pm$ 5.9) cM from *mo*, p236 is 9.8 ($\pm$ 5.9) cM from *er*, p254 is 6 ($\pm$ 1.9) cM from *Fw* and *Ascochyta pisi* race C major gene is either between p227 and p105 or between p105 and p164-3 on chromosome 4. A three-point test could not map this gene more accurately. This is probably due to the quantitative nature of Ascochyta blight resistance, which did not allow us to infer unambiguously the genotypes from the phenotypes of the plants.

Quantitative trait analysis

Quantitative trait data were checked for deviation from normality. For Ascochyta blight resistance measured on F_3 plants, a highly significant deviation was observed (Fig. 3). This deviation is due to the large number of fully resistant families (23), a result which may be clarified by the scoring being performed only 10 days after the beginning of the infection. In the subset of families with at least 1 susceptible plant, the distribution is normal. The heritability (narrow sense) of Ascochyta blight resistance computed from the complete data was 0.81; computed from truncated data it was 0.21 (i.e. without the fully resistant families). The search for QTL has been performed with both complete and truncated data. Significant skewness was found for the number of nodes measured in the field. However, the deviation was weak and could have had only a moderate effect on the reliability of the statistical tests. None of the other variables showed deviation from normality. In the absence of F_3 values, heritability was not computed for developmental traits. We observed highly significant correlations between height and number of nodes ($r = 0.55$; $P < 0.001$), number of nodes and flowering earliness ($r = 0.77$; $P < 0.001$) and flowering earliness and height ($r = 0.36$; $P < 0.01$). More unexpected, we found a significant

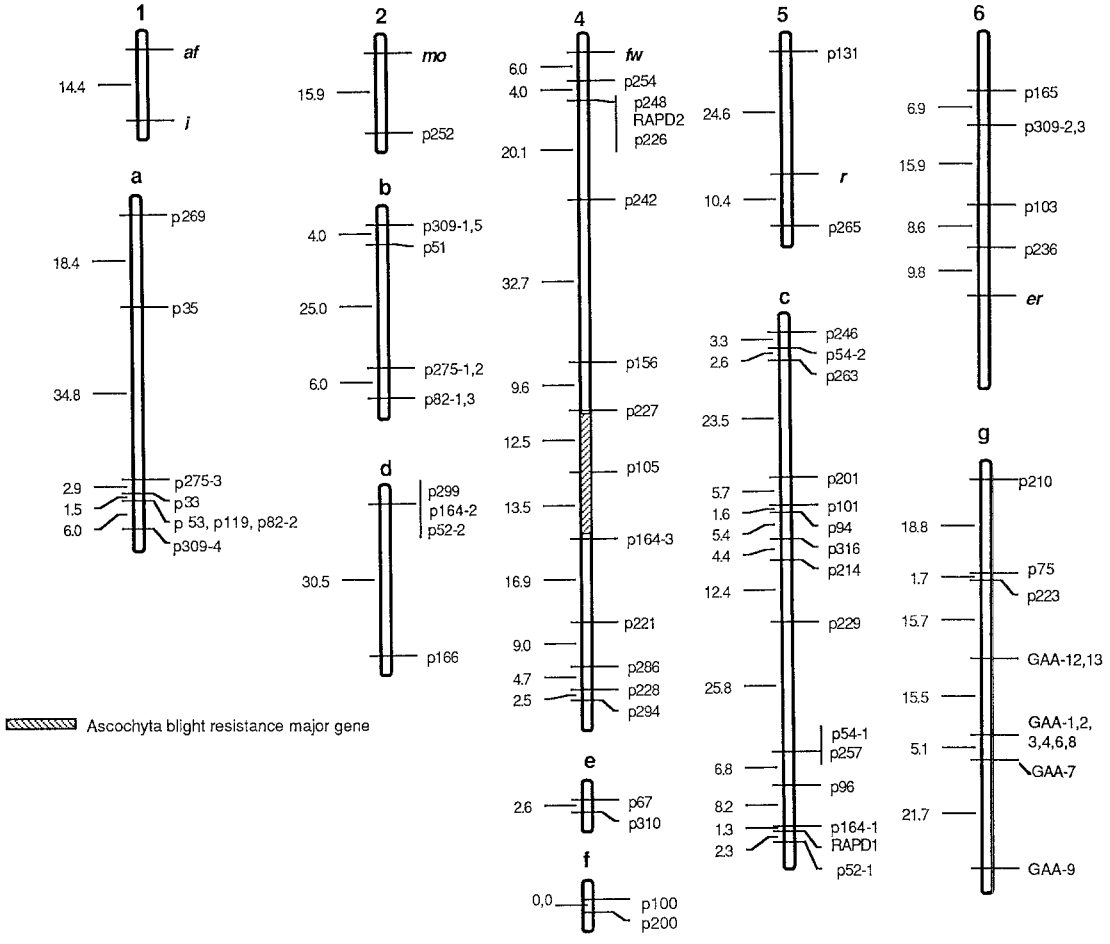


Fig. 2 Genetic linkage map of pea constructed with MAPMAKER. The genes and markers are given on the *right side* of the chromosomes; map distances are given in cM on the *left side* of the chromosomes. The letters *a–g* at the *top* of linkage groups are for groups that could not be assigned to chromosomes

negative correlation between number of nodes measured in the field and Ascochyta blight resistance ($r = -0.26$; $P < 0.01$).

Quantitative trait loci

Marker loci found to be significantly correlated ($P < 0.01$) with quantitative traits when using ANOVA are listed in Table 2. Associations were found between markers and all of the traits under study. In many cases significant loci were in clusters, which may be due to the presence of only one QTL localized within the cluster. Accordingly, we considered that ANOVA detected 12 chromosomal regions that contributed to quantitative traits, each one identified by the marker locus that displayed the highest R^2 value (proportion of variation of the trait explained by the polymorphism at the marker locus). MAPMAKER-QTL (LOD > 2.2) confirmed these results in all cases but one (p248 on chromosome 4) (Table 3). The QTLs are represented on the genetic maps of Figs. 4 and 5. The sum of the R^2 computed from ANOVA and the cumulative R^2 given by MAPMAKER-QTL were in the same order of magnitude, but the individual R^2 were not (Tables 2 and 3). Not only are the values calculated with MAPMAKER-QTL higher than those calculated with ANOVA for all QTLs, but there is a correlation between the R^2 values and the linkage distances that can only be an artifact. Hence, when comparing

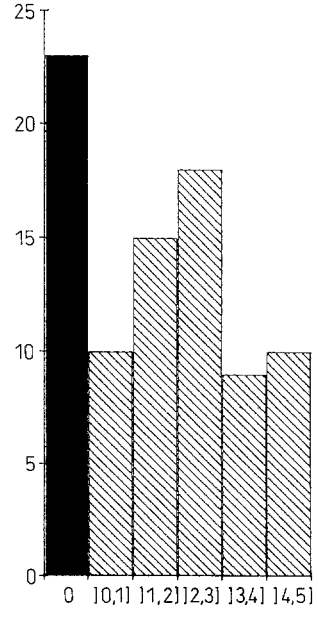


Fig. 3 Distribution of the Ascochyta blight resistance in the segregating population produced from the F_1 between 'Erygel' (susceptible) and '661' (resistant). *Abscissa* notation from 0 (fully resistant) to 5 (the most susceptible)

the individual effects of the QTLs, we have only considered the ANOVA values.

Developmental traits

For the plants grown in the greenhouse, a marker locus (p275, on linkage group b) was found to be significantly associated with plant height, flowering earliness and number of nodes, with R^2 values of 0.18, 0.42 and 0.43, respectively. On chromosome 6, a QTL (or QTLs) common to earliness ($R^2 = 0.18$) and number of nodes ($R^2 = 0.15$) was detected close to *er*.

For the plants grown in the field, where only the number of nodes was measured, an association was also found on group b, with an R^2 of 0.45 for the marker p82, which is tightly linked to p275. A QTL for the number of nodes was detected in the region of *er* only when using a less stringent threshold was used ($P < 0.05$) (data not shown), and two additional QTLs were found, one on chromosome 2 ($R^2 = 0.14$), the other on the linkage group c ($R^2 = 0.16$).

These results were confirmed using interval mapping (Table 3). On linkage group b, the QTL with large effects on height, earliness and number of nodes was found to map 4 cM from p275, with LOD scores of 2.6, 6.9 and 7.0, respectively. The QTLs common to earliness and number of nodes detected on chromosome 6 are close to p103, with LOD score values of 4.2 and 2.5, respectively. For the plants grown in the field, we detected three QTLs for the number of nodes in the same regions as those found with ANOVA: on chromosome 2 close to p252 (LOD 2.6), on linkage group b close to p275 (LOD 10.0) and on linkage group c close to p263 (LOD 2.2) (Fig. 5). Thus, two QTLs accounted for 59% (ANOVA) to 69% (MAPMAKER-QTL) of the variation in earliness, and two QTLs (possibly the same–see below) accounted for 58% (ANOVA) to 79% (MAPMAKER-QTL) of the variation in the number of nodes measured in the greenhouse. In the field, three QTLs accounted for 56% (MAPMAKER-QTL) to 75% (ANOVA) of the variation in the number of nodes (Tables 2 and 3).

Not all of the QTLs conformed to the parental phenotype. While 'Erygel' is taller than '661', it has an allele for "small-

Table 2 Significant associations between quantitative traits and marker loci detected using ANOVAs (R^2 percentage of phenotypic variation explained by the marker locus, F value from the Fisher test, a additive effect, d dominance effect, d/a ratio of the dominance to additive effects for codominant markers. The sign + or − indicates that the allele which increases the trait value is in the '661' or in the 'Erygel' parent, respectively)

Trait	Marker	R^2	F	a	+/−	d	d/a	Chromosome or linkage group
Results on the F_2 plants grown in the greenhouse (72 F_2 plants)								
Plant height	p275	18.5	5.34**	7.07	+	3.30	0.47	b
		$\Sigma R^2 = 18.5$						
Flowering earliness	p103	14.6	11.15**	nd	−	nd	nd	6
	p236	15.4	10.40**	nd	−	nd	nd	6
	er	17.8	6.81**	nd	−	nd	nd	6
	p275	41.6	17.16***	0.79	+	0.80	1.01**	b
		$\Sigma R^2 = 59.4$[a]						
Number of nodes	p103	14.9	11.49**	nd	−	nd	nd	6
	p236	9.9	6.33**	nd	−	nd	nd	6
	er	13.0	4.77**	nd	−	nd	nd	6
	p275	42.7	17.92***	2.26	+	0.80	0.35	b
		$\Sigma R^2 = 57.6$[a]						
Results on F_2 plants grown in the field (102 F_2 plants)								
Number of nodes	p252	14.2	6.5**	0.26	−	1.09	4.13**	2
	p275	36.7	18.8***	1.34	+	0.50	0.37*	b
	p82	45.0	24.6***	1.68	+	0.36	0.21*	b
	p263	16.0	5.7**	0.67	+	−0.44	−0.66	c
		$\Sigma R^2 = 75.2$[a]						
Ascochyta blight: complete data	*af*	21.5	11.4***	nd	−	nd	nd	1
	p156	43.3	21.4***	1.61	−	0.22	0.13	4
	p227	45.5	28.8***	1.64	−	−0.15	−0.09	4
	p105	43.9	27.8***	1.49	−	−0.37	−0.25	4
	p164	13.0	8.4**	nd	−	nd	nd	4
		$\Sigma R^2 = 67.0$[a]						
Truncated data	*af*	17.2	6.2**	nd	−	nd	nd	1
	p248	16.1	5.1**	nd	−	nd	nd	4[b]
	p227	29.7	10.4**	1.29	−	0.07	0.05	4
	p105	30.0	10.9***	1.17	−	0.05	0.04	4
	p236	27.1	10.1**	nd	+	nd	nd	6
		$\Sigma R^2 = 74.3$[a]						

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

[a] When linked marker loci displayed significant associations, only the marker locus with the highest R^2 value was taken into account in the calculation of the sum of the R^2

[b] This QTL, independent from the other QTL of chromosome 4, is not considered in the sum of R^2 because it is not detected when using MAPMAKER-QTL (see text)

[c] nd, Non-determined (dominant markers)

Table 3 QTLs detected using MAPMAKER-QTL with a minimum LOD score threshold of 2.2. $R^2, a, d, d/a$ and $+/-$, same as in Table 2, *nd* Non-determined (dominant markers)

Trait	Interval (cM from the left marker)	R^2	LOD	a	+/−	d	d/a	Chromosome or linkage group
Results on the F_2 plants grown in the greenhouse (72 F_2 plants)								
Plant height	p275/p82(4)	21.0	2.6**	8.1	+	3.0	0.37	b
		$\Sigma R^2 = 21.0$						
Flowering	p309/p103(22)	74.2	4.2	1.3	−	nd	nd	6
earliness	p275/p82(4)	45.2	6.9	0.9	+	0.7	0.78	b
		$\Sigma R^2 = 69.1$[a]						
Number	p103/p236(0)	16.3	2.5	1.4	−	nd	nd	6
of nodes	p275/p82(4)	46.2	7.0	2.3	+	0.9	0.39	b
		$\Sigma R^2 = 79.3$[a]						
Results on F_2 plants grown in the field (102 F_2 plants)								
Number	mo/p252(18)	14.8	2.6	0.3	−	1.1	3.67	2
of nodes	p275/p82(2)	50.5	10.0	1.7	+	0.9	0.53	b
	p263/p201(0)	11.2	2.2	0.7	+	nd	nd	c
		$\Sigma R^2 = 55.9$[a]						
Ascochyta blight:								
complete data	*af/i* (8)	38.0	4.9	1.1	−	nd	nd	1
	p227/p105(8)	59.8	12.5	1.7	−	−0.5	−0.29	4
		$\Sigma R^2 = 60.7$[a]						
Truncated data	*af/i*(16)	38.3	2.8	0.5	−	nd	nd	1
	p227/p105(8)	44.7	5.6	1.4	−	−0.2	−0.14	4
	p236/*Er*(4)	57.7	3.9	0.9	+	nd	nd	6
		$\Sigma R^2 = 70.8$[a]						

[a] Cumulative R^2 given by MAPMAKER-QTL

ness" for the QTL close to p275 on group b. Similarly 'Erygel' is earlier and has fewer nodes than '661', although it has an allele for high values of these traits on chromosome 6. The same is observed for the QTL located on chromosome 2 that affects the number of nodes measured in the field.

The major QTL(s) located on linkage group b, which control(s) the three developmental traits measured in the greenhouse, displayed dominance, with the "high" allele being dominant over the "low" allele; a highly significant dominance value was observed for earliness. Significant partial domi-

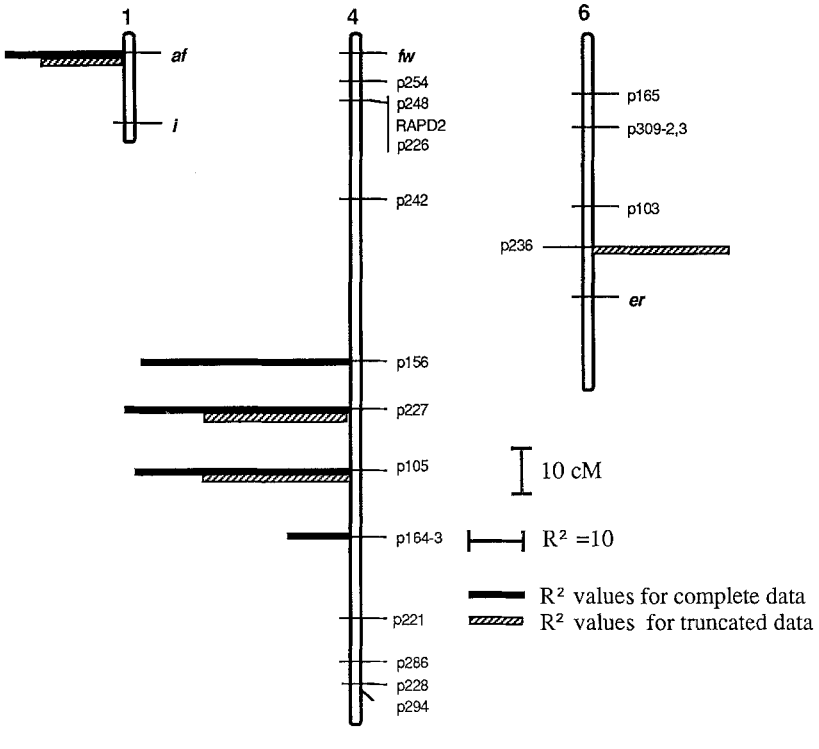


Fig. 4 Marker loci significantly correlated with resistance to Ascochyta blight in the F_2 progeny between the pea varieties '661' and 'Erygel'. The *length* of the *horizontal lines* is proportional to the R^2 values; *black lines* R^2 values obtained with the complete data; *grey lines* R^2 values obtained with the truncated data, i.e. where the fully resistant plants were not taken into account. The QTL found on the chromosome 6 displayed the opposite effect relative to the parental phenotypes

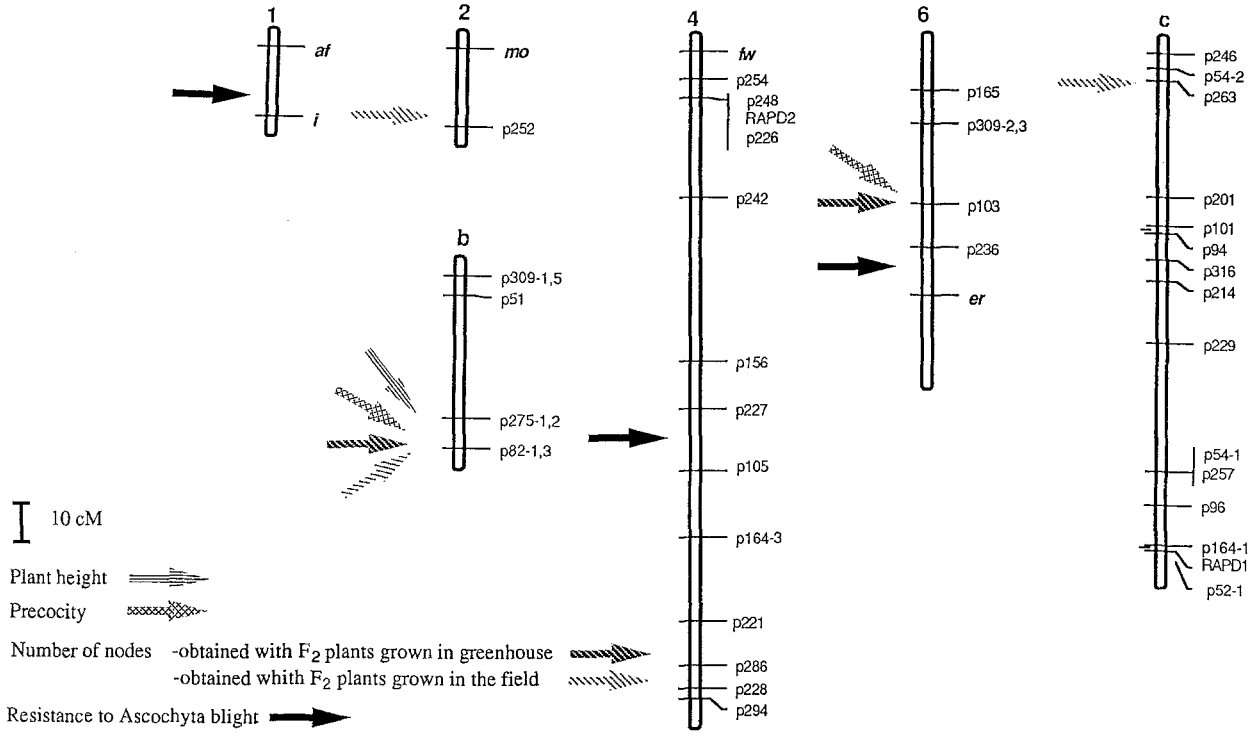


Fig. 5 QTLs detected for the developmental and pathological traits under study. *Arrows* point to the maxima given by MAPMAKER-QTL

nance was observed in this region for the number of nodes measured on plants grown in the field. The QTL found on chromosome 2, which contributes to the number of nodes for field-grown plants, displayed highly significant, non-additive inheritance, the factor that increases the number of nodes being dominant (Table 2).

Ascochyta blight resistance

Two chromosomal regions were found to be associated with Ascochyta blight resistance when ANOVA was used on complete data: *af*, on chromosome 1, displays an R^2 of 0.21, while four linked markers on chromosome 4 were significant, with a maximum R^2 of 0.45 for marker p227. With truncated data (i.e. without the fully resistant families, see Quantitative trait analysis section), both regions were detected, with a R^2 of 0.17 and 0.30, respectively, but two additional markers proved to be significant: p248 on chromosome 4 (unlinked to the above-mentioned group), with an R^2 of 0.16, and p236 on chromosome 6, with an R^2 of 0.27 (Table 2). Actually, these last two QTLs were suspected from the ANOVAs carried out on complete data, but in these they were significant only at the 0.05 level and displayed an R^2 around 0.10. In all, the two significant regions found from the complete data accounted for 67% of the variation, and the four regions detected from truncated data accounted for 74% of the variation.

Surprisingly, one QTL displayed an unexpected parental genotype. While '661' is considered to be fully resistant to Ascochyta blight, it is homozygous for the susceptible factor for the QTL located on chromosome 6.

Interval mapping confirmed that there is a major QTL on chromosome 4 between p227 and p105 (LODs 12.5 and 5.6 for complete and truncated data, respectively) and another one between *af* and *i* on chromosome 1 (LODs 4.9 and 2.8 for complete and truncated data, respectively). With the truncated data, a QTL was also detected on chromosome 6 between p236 and *er* (LOD 3.9), but no significant effect was found in the p248 region of chromosome 4 (Table 3). Thus, we consider that three main QTLs are involved in the resistance to this disease in this progeny, one of them with a reverse effect compared to the behavior of the parent. The major QTL of chromosome 4 displayed codominance. Inheritance could not be determined for the QTLs of chromosomes 1 and 6 due to dominance at the linked marker loci.

Discussion

Restriction fragment length polymorphism

Fifty percent of the probes used detected polymorphism between the two pea parental lines with two restriction endonucleases (*Eco*RI and *Hin*dIII). Higher levels of polymorphisms have been observed in rice (McCouch et al. 1988) and corn (Helentjaris et al. 1985). In *Arabidopsis*, 48% of the clones revealed polymorphism with three restriction enzymes (Chang et al. 1988). Polymorphism has been found to be much lower in tomato (Bernatzky and Tanksley 1986; Tanksley and Bernatzky 1987) and in hexaploid wheat (Chao et al. 1989). For pea, the level of polymorphism was surprisingly high if we consider the pea to be a self-pollinating species. In tomato, the facultatively outcrossing species *Lycopersicon chmielewskii* was observed to be much more polymorphic than the self-pollinating species *L. esculentum* (Helentjaris et al. 1985). The self-incompatible species *L. pennelli* was also reported to be

more polymorphic than the cultivated tomato. The high level of polymorphism that we have observed in pea may also be the reflection of the very distinct origins of the parental lines, 'Erygel' and '661'.

The alterations in fragment length detected by restriction endonucleases could be the result of either point mutations or deletion-insertion events. Point mutations are detected only if the mutations occur within the recognition site of the restriction endonuclease. In contrast, deletion-insertions are detected by all restriction endonucleases that have recognition sites flanking the change in the DNA. Among the 166 probes, 40 revealed polymorphism with both enzymes, suggesting that there is significant insertion-deletion polymorphism in pea. This type of analysis was done in *Triticum tauschii*, and the results suggested that both deletions-insertions and point mutations contribute equally to the generation of RFLPs (Gill et al. 1991).

Linkage map

There are linkage maps for pea based on morphological, physiological and pigmentation characters (Blixt 1975), on isozyme data (Weeden and Marx 1987) and the combination of morphological, isozyme and DNA markers (Weeden and Wolko 1990). Ellis et al. (1992) presented a linkage map that included DNA markers and numerous markers already localized in the previous maps; their linkage map comprised 151 markers and spanned approximately 1700 cM. The partial map of pea described here includes morphological characters, disease resistance genes and DNA markers. For each disease, powdery mildew, Fusarium wilt, pea common Mosaic virus and Ascochyta blight, we found at least one molecular marker linked to resistance. More markers are needed to get a more complete linkage map. The identification of closer linkages between molecular markers and resistance genes, for example using the method of bulked segregant analysis (Michelmore et al. 1991), could lead to the production of highly reliable tests for predicting disease resistance at early stages of development. It may also facilitate the cloning of disease resistance genes by chromosome walking.

Highly repeated DNA sequences are thought to be dispersed throughout all of the genome. For example, Lee et al. (1990) described in pea a repeated sequence belonging to the *copia*-like class of retrotransposons and showed that this sequence can be used to follow simultaneously the segregation of many loci dispersed in the genome in conventional RFLP analysis. The results obtained with the microsatellite probe $(GAA)_6$ are unexpected since no segregation was observed between most of the polymorphic bands. In addition, the loci included in the map are located on the same linkage group within 42 cM of each other (Fig. 2). Thus, the $(GAA)_n$ tandem repeats may be dispersed through the genome, but the polymorphic regions do not seem to be randomly distributed.

Developmental traits: genetic basis of correlations

Our pea linkage map, though partial, allows us to explain most of the phenotypic variation for number of nodes and earliness, since for these traits the overall R^2 given by MAPMAKER varied from 56% up to 79%, with only two or three QTLs. For plant height, we can explain no more than 20% of the variation, with one QTL. Field and greenhouse data resulted in partly different sets of QTLs for the number of nodes, but the major QTL close to p275 was detected in both environments. Thus, it seems that they are only moderate genotype × environment interactions for this trait.

Height, earliness and the number of nodes appeared to be positively correlated in the progeny. Such a relationship is common in pea (Wellensiek 1925). However, in the parents we used, height displays the opposite behaviour, since with fewer nodes and fewer days to flowering than '661', 'Erygel' is taller than '661'. The QTL analysis shed light on this apparent inconsistency. A possible QTL common to these traits (or linked QTLs) was found on linkage group b. More than 40% of the variation in number of nodes and earliness was explained by this region, and the effect of substituting the factor from 'Erygel' was about 4.5 nodes and 6.4 days (data not shown). In contrast, this region affects the height moderately (R^2 around 20%, with a 14-cm difference mediated by this region) and in a direction consistent with the observed correlation. This QTL is likely to be responsible for the positive correlation between these traits, and for the higher correlation observed between earliness and number of nodes than between height and the other traits. As no QTL that followed the parental behaviour was detected for height, one can suppose that such QTL(s) were in regions of the genome not covered by the markers and/or were below our threshold of detection. The latter hypothesis is supported by the observation, from a comparison of various pairs of isogenic lines, that *af* slightly decreases the height of the plants with no detectable effect on the number of nodes (R. Cousin, unpublished results): as '661' is *af/af* and 'Erygel' is *Af/Af*, we could have expected a minor QTL in the *af* region, with an effect consistent with the behaviour of the parent.

In addition to the region on group b, a region on chromosome 6 explained around 15% of the variation for both number of nodes and earliness. Besides physiological reasons [late-flowering plants may have large number of nodes (Wellensiek 1925)], we favour the hypothesis of the pleiotropic action of genes rather than linked QTLs: even though the confidence intervals of mapping positions are large, it seems unlikely that two traits share by chance more than one QTL (for example with a large confidence interval of 25 cM in a genome of 1,000 cM, the probability that two physiologically unrelated traits display two apparent common QTLs is only 1/780). Thus, our results suggest that the number of nodes and earliness have, to a large extent, a common genetic control mechanism.

The negative correlation between the number of nodes measured in the field and Ascochyta blight resistance could be accounted for by a possible common QTL on chromosome 6, whose effect for both traits is reversed relative to the expected parental contributions. However, no convincing physiological explanation can be given, and a genetic relationship can be suspected, but should be verified with other crosses.

There are very few examples of applying the QTL approach to the genetics of oligogenic disease resistances in plants. Heun (1992) was able to explain about 20% of the variation of powdery mildew resistance of barley with two QTLs. In our case, high overall R^2 values were obtained. With complete data, 61% (MAPMAKER-QTL) to 67% (ANOVA) of the variation of the resistance was explained by two QTLs. With truncated data, 71% (MAPMAKER-QTL) to 74% (ANOVA) of the variation of the resistance was explained by three QTLs (ignoring the QTL detected by ANOVA alone). It is worth noting that since the F_2 plants were given the mean values of their F_3 progeny, the R^2 values account for more genetic variation than if they were computed from the per se F_2 values.

The major QTL mapped on chromosome 4 ($R^2 = 0.45$) corresponds to the gene located by considering resistance as a qualitative trait. The polymorphism of this QTL changes the resistance from 3.53 (mean value of the plants homozygous for the susceptible factor) to 0.13 (mean of the plants homozygous for the resistant factor), i.e. it accounts for a large range of the resistance (noted from 0–5) (data not shown). The existence of this major QTL is consistent with the observation of Cousin (1974) as it displays intermediate dominance. Another QTL, located on chromosome 1 in the *af-i* region, was detected from complete ($R^2 = 0.21$) as well as truncated ($R^2 = 0.17$) data. It may correspond to *Rap 2*, the major dominant gene described by Darby et al. (1985) in the *i* region. The location does not appear to be exactly the same (*Rap 2* is beyond *i* in the *af-i* segment, while our QTL would be within the interval), but the mapping accuracy is not sufficient to propose the presence of two different factors in this region. A QTL on chromosome 6 with a non-significant effect when working on complete data became highly significant on truncated data ($R^2 = 0.27$), since eliminating the resistant plants resulted in decreasing the variance of the marker locus genotypic classes (residual of the ANOVAs). This QTL changes the mean resistance from 3.53 to 1.63 (in the truncated distribution). Interestingly, it displayed the opposite effect relative to the parental phenotypes. In combining the appropriate alleles, it would be possible to create a pea variety more resistant to *Ascochyta pisi* race C than var '661', one of the most resistant known so far. Markers flanking this QTL could be used for such a purpose.

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